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ANNOUNCEMENT

It is proposed to issue Volume VIII of *The Journal of General Physiology* as a memorial to its founder, Dr. Jacques Loeb. This volume will contain papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life. The volume will appear simultaneously with Volume IX, and publication will commence September 18, 1925.

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PHYSIOLOGICAL ONTOGENY.

A. CHICKEN EMBRYOS.

II. CATABOLISM. CHEMICAL CHANGES IN FERTILE EGGS DURING INCUBATION. SELECTION OF STANDARD CONDITIONS.*

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INTRODUCTION.

Kirchoff (1) stated that the object of mechanical science was "to describe completely and in the simplest manner the motions which occur in nature," and so, from the standpoint of energetics, life may be viewed as a mechanism and described in terms of the absorption and elimination of energy. In the absence of external work, the developed energy resolves itself into heat, and as such may accordingly be estimated directly or indirectly, so it seems, by measuring the exchange of the respiratory gases. For a proper understanding of these processes one must know the quantity and quality of material ingested in a given unit of time, and what part of the absorbed potential energy is retained and what part converted and dissipated as heat. The experiments to be presented in this paper deal with the latter factor since they involve determinations of the amount of carbon dioxide eliminated by embryos of successive incubation ages. These tests were supplemented by chemical analyses before and after incubation. The chemical analyses were to serve also as a preliminary to a subsequent study of the changes in the constitution of the embryo with age, so that a comparison might be made of the concentrations of the more important substances inside and outside the embryo.

It is important to know the amount of (1) fuel (carbohydrate, protein, and fat) burned in embryonic metabolism, (2) water lost by

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* An article of an introductory nature dealing with the general point of view from which these experiments were undertaken will be published elsewhere.

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evaporation, and (3) solid substance, if any, contributed to the developing embryo by the egg shell. We assume that the weight of all other non-oxidizable substances within the egg remains constant throughout incubation.

Preparatory to these and subsequent experiments it was necessary to establish suitable incubation conditions, the maintenance of which would increase the probability that development proceeded at approximately the same rate in each egg. With this object a few observations have been made upon the atmospheric temperature and humidity as variables modifying the constitution of the whole egg during incubation.

The Source and Control of the Experimental Material.

Unless it is stated to the contrary the management of the fertile egg as now described applies to all subsequent experiments of this series. The eggs are those of White Leghorn hens 9 to 18 months of age raised and kept on the same farm. They are collected several times a day, refrigerated at about 55°F. during the night, shipped the next morning and delivered cold at the laboratory the following day. Approximately 2 days after they are laid they are incubated in the laboratory.

There are numerous variable factors which can modify the constitution of the egg as well as the rate of development of the blastoderm (2). Investigations at agricultural experimental stations have revealed that the amount of yolk, albumin, and water, as well as the thickness of the shell, vary according to the season, diet, and general condition of the bird (3). Variations in the relative proportion of these substances may also occur as a result of individual or chance happenings, such as the velocity of the egg's passage down the oviduct whence the albumin is derived, the time intervening between laying and collecting, the humidity and temperature of the surrounding atmosphere after laying and during transportation. As the initiation of ontogenesis by fertilization occurs in the upper reaches of the oviduct and as the process of egg formation in the hen usually takes from 18 to 30 hours, the development of the blastoderm with differentiation of the three germ layers has already taken place by the time the egg is laid. Variations then in the duration of the egg's stay within the

reproductive organs of the hen so affects development that no two embryos will be exactly the same age at the time of laying. Poultry-men recognize what are called body-heated eggs, that is to say, eggs held in the body of the fowl for 2 or more days. Since temperature conditions are suitable, development will be found to have progressed further than usual in such eggs. If, after laying, the temperature is ever raised as high as 72°F. it is said that growth of the blastoderm will commence and proceed slowly. Likewise, if a hen sits on the egg for a time after laying, the stage of development at the commence-ment of incubation will be more advanced than the average. These, then, are some of the factors, which in our experiments were more or less variable. Their importance could only be minimized by the accumulation of sufficient data, that is to say, by the statistical method.

In a few of the subsequent experiments the eggs were kept in a Lo-Glo incubator at about 39°C. Once a day they were taken out into the laboratory and rolled. Later, when a constant temperature room was built, a water-jacketed copper box provided with a constant inlet of warm fresh air was used as an incubator. The maximum variation of temperature within the box was ± 0.4.[1] The eggs were turned once or twice a day and weighed at less frequent intervals. All manipulations were done in the room where the humidity was less than in the box, but the temperature was approximately constant at 38.8° ± 1.0°C.[1]

Weight and Surface Area of Eggs.

The average weight of over 500 eggs before incubation was found to be 57.8 gm. It is stated in the literature that during incubation there is a loss of weight most of which can be accounted for by the evaporation of water. The extent of the loss was usually very vari-able, but there were found no analyses of the factors which determined it. As the amount of evaporation and gaseous exchange in the egg must be a function of its surface rather than of its mass, it was con-sidered advantageous to find a convenient method for measuring the surface area. Three simple indices were tried for each of twenty-seven

[1] ± 0.4 and ± 1.0 refer to the maximum range.

eggs: (1) the projected surface. To obtain this a piece of apparatus was constructed to hold the egg firmly in position resting on a flat sheet of paper. The greatest circumference of the egg was then drawn with a pencil held perpendicularly in a block and the enclosed area representing the largest cross-section of the egg was measured with a planimeter; (2) the product of the maximum length and width; and (3) the two-thirds power of the weight $(W^{\frac{2}{3}})$ of the unincubated egg. An appropriate constant by which to multiply the average result of each method was found by comparing each result with the actual surface as measured with adhesive plaster. By comparing the average errors in the measurement of the surface by each formula, it was pos-

TABLE I.

Measured and Calculated Egg Shell Surfaces Compared.

	Average of eggs (Nos. 1 to 27) ± standard error.[*]
Measured surface, *sq. cm*	75.00 ±0.71
Weight, *gm*	56.94 ±0.82
$W^{\frac{2}{3}}$	14.79 ±0.14
$K = \dfrac{\text{measured surface}}{W^{\frac{2}{3}}}$	5.07 ±0.10
Average deviation of individual, calculated surface from observed surface, *sq. cm*	±1.1

[*] Standard error $= \dfrac{\text{Standard deviation}}{\sqrt{\text{No. of observations}}}$.

sible to estimate approximately which method was the most accurate. The third method for which the equation is $S = 5.07\, W^{\frac{2}{3}}$ gave the most satisfactory results. The average error was ± 1.1 sq. cm. as compared to ± 1.9 and ± 1.6 sq. cm. for the first and second methods respectively. For this reason and because the method dispensed with all measurements except the weight it was selected. (Table I.) It may, of course, be conveniently represented graphically, so that, knowing the weight, the value for the surface can be read off immediately.

Using this formula to obtain the surface area a very slight correlation was found between surface and the amount of weight lost during incubation when other factors such as temperature and humidity

were kept constant. It was then surmised that perhaps the thickness of the shell might be a factor. The thickness of the shell was estimated by finding the weight per sq. cm. $\left(\dfrac{\text{Weight of shell}}{S}\right)$. This approximation for the thickness was compared with the average daily weight lost per sq. cm. It was found that the two varied inversely and that the correlation in this case was more definite than in the case of the egg surface. These estimations, however, are necessarily crude since-the thickness of the shell is not uniform. An egg with a heavy shell which has very small rarefied areas may lose more weight than one having a lighter shell of uniform thickness. This was shown to be a fact by making very small cracks in the shell, not sufficient to interrupt development. The result was that eggs so treated lost 50 to 100 per cent more weight per day than the average.

The conclusion was reached that, although the area and thickness of the shell were among the determining factors in the loss of weight during incubation, the thickness, which seemed the more important of the two, could not be measured with any degree of exactitude, and that, at least for present purposes, if care were taken to choose eggs of approximately uniform size and shell thickness, both functions could be neglected.

The Weight of the Shell during Incubation.

There seems at last to be general agreement about the fact that the shell, the most important element of which is calcium carbonate, loses weight during incubation. The discussion on this point which originated when William Prout (4) first affirmed in 1822 the passage of earthy carbonates into the embryo from the shell, seems to have been largely the result of disregarding the great variability of eggs in almost all their characters. Whatever method is used necessarily involves estimations of the total shell weight or the amount of calcium in the shell or egg contents before and after incubation. As the same eggs cannot, of course, be used for both determinations, and as egg shells before incubation vary at least as much as between 4.5 gm. and 7.7 gm. in weight, or between 7.7 per cent and 11.9 per cent of the total egg weight. it will not be evident whether there is a loss of such a small fraction as 0.3 gm. of shell substance during incubation unless a care-

ful statistical study is made.   None of the previous investigators have chosen to do this.

In 1908 Tangl (5) introduced an improvement when he limited himself to a comparison of eggs from the same hen on a standard mineral diet.   He believed that his figures showed a loss of 0.3 to 0.4 gm. of shell weight during incubation.   His general conclusions have been confirmed by Carpiaux (6) and again more recently by Plimmer and Lowndes (7).

Tangl weighed and analyzed eight dried egg shells before incubation and fifteen after incubation.   From his data it may be estimated that the shell weight was 9.64 ± 0.09 per cent[2] of the total egg weight before

TABLE II.

Weight of Shell in Terms of Total Egg Weight before and after Incubation.

Incubation age.	No. of eggs weighed.	Average weight of eggs before incubation.	Average weight of shell.	Average percentage weight of shell.	Average surface area. $S = 5.07 W^{\frac{2}{3}}$	Average weight of shell per sq. cm.	Shell weight in an egg of average weight (57.8 gm.).	Average loss of shell weight.
days		*gm.*	*gm.*		*sq. cm.*	*gm.*	*gm.*	*gm.*
0	35	58.81	5.767	9.81 ±0.11	76.6	0.0753	5.670	
17	25	59.09	5.590	9.46 ±0.14	76.9	0.0727	5.468	0.202
18	16	59.28	5.609	9.46 ±0.09	77.1	0.0728	5.468	0.202
19	22	59.62	5.580	9.36 ±0.12	77.4	0.0721	5.410	0.260

incubation, whereas after incubation it was 9.08 ± 0.10 per cent, the difference between these two averages being 0.56 ± 0.13 per cent. Tangl used a different variety of fowl (Plymouth Rock) from ours in his experiments, and took all his eggs from three hens.   We, on the other hand, used eggs from White Leghorn hens and desired average figures representative of the whole flock.

For our purposes it is necessary to know only approximately to what degree, and at what rate the solids of the egg are augmented by the addition of shell constituents.   To learn these facts series of egg shells were weighed before and after 17, 18, and 19 days of incubation, respectively (Table II).   The weights were obtained after drying the

[2] Unless otherwise stated the designation ± a number refers to ± the probable error; *i.e.*, $0.6745 \dfrac{\text{standard deviation}}{\sqrt{\text{No. of observations}}}$.

shells in an oven. Before incubation the shells were 9.81 ± 0.11 per cent of the total egg weight; whereas after 17, 18, and 19 days of incubation they were 9.46 ± 0.14, 9.46 ± 0.09, 9.36 ± 0.12 per cent, respectively, of the initial egg weight, the difference being consequently 0.35, 0.35, and 0.45 per cent. This might be compared to the difference found by Tangl after incubation, namely, 0.56 per cent. The greater loss of weight in his experiments is probably due to the fact that his incubated eggs were examined later, after 20 days of incubation. Tangl obtained greater differences in a later but smaller set of weighings (8).
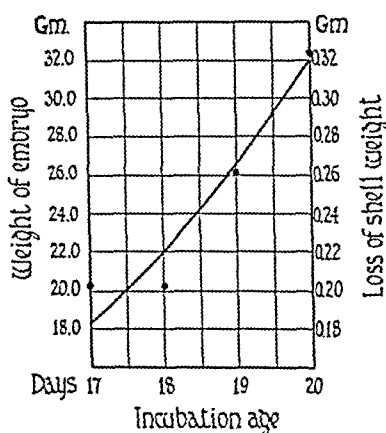
FIG. 1. A comparison of the weight of the embryo (heavy line) as determined by the formula $W_e = K\ t^{3.6}$ and the average loss of shell weight (black circles (●)) as calculated from a series of weighings (Table II) when expressed as functions of the incubation age.

The differences are not great as judged by the criteria of statistical theory, and inasmuch as our results are comparable to those of Tangl, who had the advantage of working with eggs from the same hen, the data may be taken to afford a basis for an approximation which would probably allow for greater accuracy in computing the changes in concentration of total solids during incubation. If there is a transfer of substance from the shell into the interior of the egg it must be known at what rate this occurs. Tangl showed a loss of 0.56 per cent in shell weight after 20 days of incubation. In an egg weighing 57.8 gm. this is equivalent to 0.324 gm. of shell substance. Using this figure

together with our own results, and plotting the loss of shell weight against the incubation age, it may be seen (Fig. 1) that the loss of weight increases with the wet weight of the embryo, the latter being represented in the figure by the curve. This fitting may be coincidental, but without the collection of a much greater number of observations it may serve as a basis for a simple approximation which would lead to a greater degree of accuracy than would be obtained by neglecting time as a function in the loss of shell weight or neglecting the latter altogether. Carpiaux, as well as Plimmer and Lowndes, found approximately 0.04 gm. of CaO in the egg contents before incubation and 0.20 gm. after incubation. Now, as the work of Tangl and Carpiaux demonstrated no change in the constitution of the shell during incubation, and as the average of eleven analyses by Plimmer and Lowndes gave the CaO as approximately 50 per cent of the total shell weight, it would seem that the loss of shell weight during incubation was about 0.32 gm., a figure which almost exactly corresponds to our own. Plimmer and Lowndes in one series determined the lime in the egg contents on consecutive days. Their average results, when multiplied by 2 to show the loss of shell weight, follow the trend of our graph, but are all somewhat lower. Since their chicks hatched later than ours, it might well have been that the embryos were larger in our series and thus more lime had been absorbed.

From the graph it is seen that for every 1.0 gm. of embryo there is a loss of 0.01 gm. of shell substance, that is to say a gain of 0.01 gm. of total solids in the egg contents.

To obtain the weight of the shell before incubation, the following formula may be used:

$$L_0 = L_t + 0.01 \, W_e \qquad (1)$$

where L_0 = the weight of shell before incubation, L_t = the weight of the shell after t days of incubation, and W_e = the wet weight of the embryo.

It will be shown in a later paper that the weight of the chick embryo between the 5th and the 19th days of incubation may be expressed by the equation

$$W_e = \frac{t^{3.6}}{1.496}$$

where W_e = the weight in mg. of the embryo, and t = the incubation age in days. For L, the weight of solids derived from the shell and added to the egg contents, the following equation may be used.

$$L = \frac{t^{3.6}}{149.6} \qquad (2)$$

These figures would be subject to a correction if it were known that some of the carbonates from the shell were transformed into carbon dioxide gas and as such passed out into the atmosphere. The metabolic determinations would likewise be affected. In the absence of the necessary data calculations have been made without correction as if the probable retention of CO_2, due to the association of calcium carbonate, upon its dissolution from the shell, with carbonic acid to form bicarbonate, was compensated by the delivery of an equal amount of CO_2 when the calcium is reprecipitated to form bone in the embryo.

The Concentration of Water, Total Solids, and Fat during Incubation.

The first experiment on the loss of weight during incubation was done while using a Freas incubator which one was required to open for other purposes several times a day. Thirty-three eggs were weighed every day throughout the incubation period as a preliminary test. There was found to be an equal distribution of fertile, unfertile, and degenerated eggs and these were all averaged together. The mean weight of all the eggs for each day was plotted against the time (Fig. 2). The diagram shows that the daily weight decrement is constant. The temperature was usually 38°C. in the incubator, but as the doors were opened periodically it dropped below this value at times. The presence of an electric fan which circulated the air over the eggs hastened evaporation. Once a day each egg in a large weighing bottle was removed into laboratory conditions for weighing. At this time it was rolled. The humidity in the incubator was not measured.

In subsequent experiments information was sought on the effect of what we considered to be the two most important variable factors affecting incubation, namely (1) temperature and (2) humidity. Since poultrymen have learned by experience that it is important to

roll eggs regularly, the surface of each egg was divided into three numbered sections by equidistant longitudinal axial lines. The eggs were placed on small rubber rings so that there should be no free motion and laid in the drawers so that their sides touched. When rolled, each egg was turned two-thirds of the way round, so that the segment number next but one pointed upwards. It was found that among the eggs rolled twice a day there was a higher percentage of live embryos than among those rolled once a day or once every other
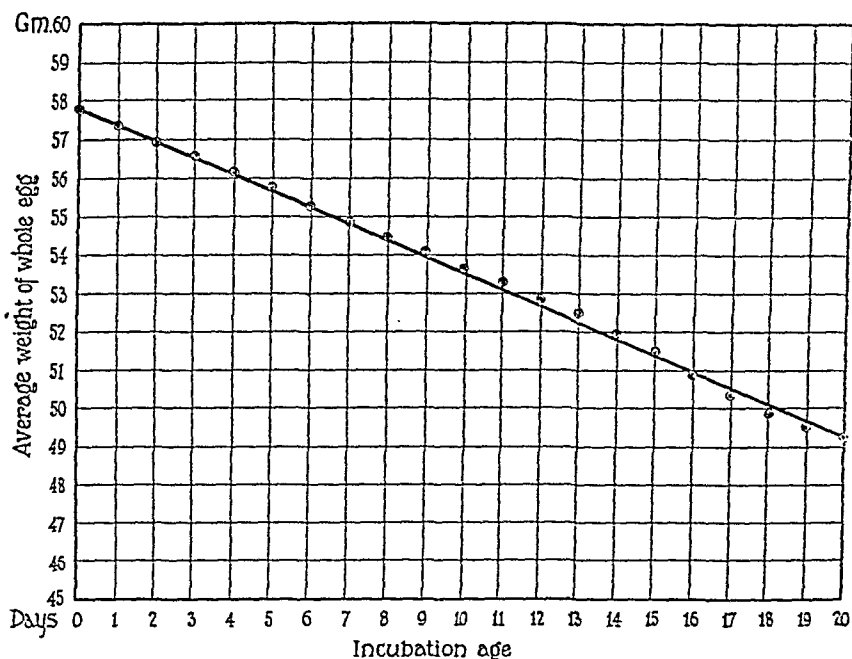
FIG. 2. The average weight of thirty-three eggs, fertile and unfertile, during the entire period of incubation.

day. But since neither the weight nor the chemical constitution of the eggs was found to vary in any regular fashion, this factor was not investigated further.

With the temperature constant three series of eggs were incubated at different concentrations of water vapor and periodically weighed. The humidity was determined by the use of dry and wet bulb thermometers. Eggs were opened on the 17th, 18th, and 19th days and the embryos therein as well as the rest of the egg contents were

analyzed for solids and fats. For the most part there were no differences in the weight lost by fertile and unfertile eggs (Table III, Fig. 3), between the 4th and 14th days of incubation the points in all cases approximated a straight line. It is therefore possible to compare the average loss of weight per day of eggs at different humidities. The
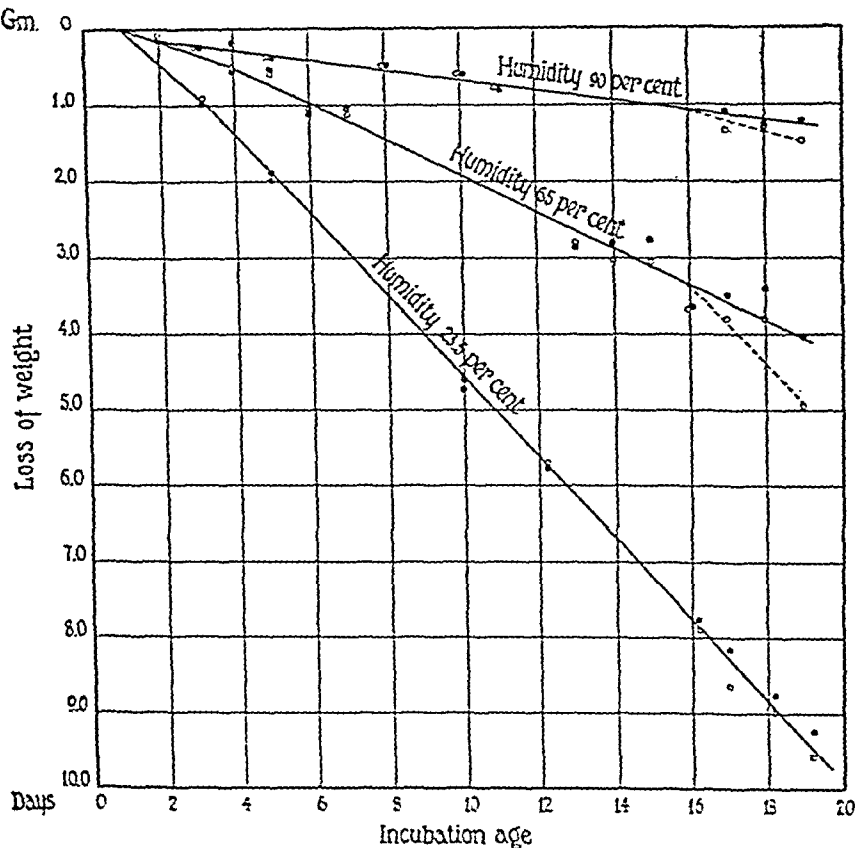
FIG. 3. The average weight of fertile (white circles (o)) and unfertile (black circles (●)) eggs, incubated at humidities of 90, 65, and 23.5 per cent respectively, equated against time (Table III).

deviations from the straight line in the middle period it was thought were due to minor fluctuations in the humidity, a factor which it was never possible to keep quite constant. The humidity of the atmosphere, apparently, determines the amount of weight lost during incubation (Fig. 4). This leads to the inference that the loss of weight

TABLE III.

Loss of Weight of Incubated Eggs under Different Conditions of Humidity.

Temperature 38.2°. Humidity 90 per cent.

Conditions	Fertile or unfertile	No. of eggs	Average weight of egg	Average weight of shell	Average loss of weight per day						Loss of weight				
			gm.	gm.	gm.	Incubation days:									
						2	3	4	5	8	10	11	17	18	19
						gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
	F.	23	57.82	5.409	0.066	0.11	0.25	0.27	0.39	0.48	0.62	0.79	1.36	1.28	1.49
	Unf.	29	60.18			0.14	0.25	0.26	0.40	0.49	0.61	0.81	1.11	1.26	1.23

Temperature 38.2°. Humidity 65 per cent.

Conditions	Fertile or unfertile	No. of eggs	Average weight of egg	Average weight of shell	Average loss of weight per day	Loss of weight										
			gm.	gm.	gm.	Incubation days:										
						4	5	6	7	13	14	15	16	17	18	19
	F.	12	60.04	5.596	0.242	0.49	0.59	1.10	1.13	2.82	3.04	3.05	3.69	3.83	3.82	4.96
	Unf.	19	57.70			0.52	0.56	1.14	1.14	2.88	2.81	2.79	3.68	3.51	3.41	4.06

Temperature 38.2°. Humidity 23.5 per cent.

Conditions	Fertile or unfertile	No. of eggs	Average weight of egg	Average weight of shell	Average loss of weight per day	Loss of weight							
			gm.	gm.	gm.	Incubation days:							
						3.2	5	10	12.2	16.2	17	18.2	19.2
	F.	8	57.14	5.375	0.533	0.96	1.91	4.61	5.71	7.93	8.66	9.01	8.89
	Unf.	8	58.25			1.00	2.00	4.75	5.79	7.78	8.19	8.78	9.26

is due to the evaporation of water. For instance, when there is 100 per cent humidity the egg loses no weight. The equation for this line is:

$$H_a = 7.5 (100 - h) \qquad (3)$$
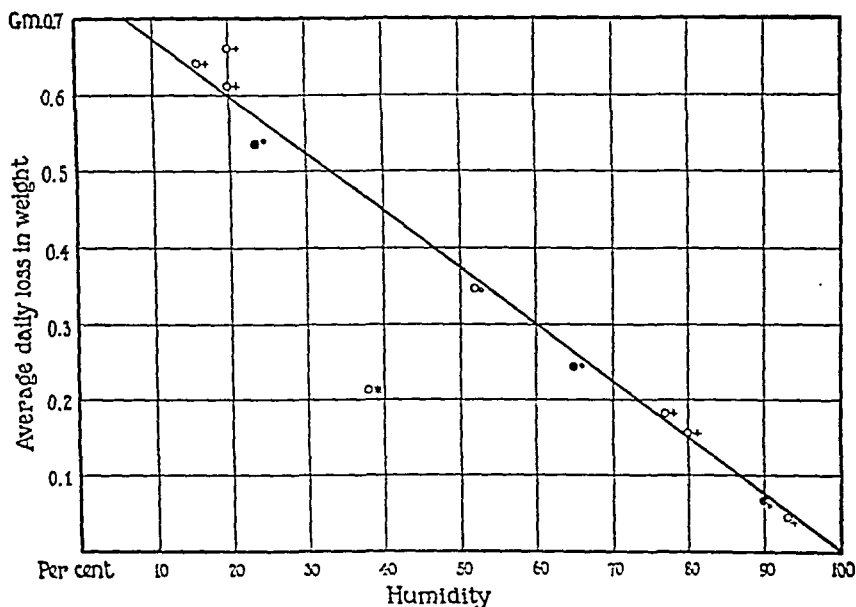
Fig. 4. The average daily loss of weight of incubated eggs as a function of the humidity. The black circles (●) represent averages of a relatively large series of eggs over the entire period of incubation; the white circles (○) are averages from a small series of eggs measured over a 2 day period. Points marked with the further notations + and ° and * refer to eggs kept at temperatures above 38.8° (+), just below 38.8° (°), and at laboratory temperature (*) respectively (Table IV).

where H_a = the average daily loss of weight (mg.) and h = humidity in per cent, and thus

$$H = 7.5 (100 - h)t \qquad (4)$$

where H = weight in mg. of the water lost by evaporation in t days of incubation.

As a confirmation of the initial findings a few shorter experiments were performed (Table IV). Each white circle (○) (Fig. 4) on the

chart represents the average loss of weight of about a dozen eggs measured over a 2 day period. The temperature was not the same in all these experiments. The points marked with a cross (+) represent experiments in which the average temperature was above, those with a circle (°) experiments in which the temperature was below 38.8°C. It appears that increasing the temperature augments the amount of weight lost by evaporation. The point far below the line in this figure was the result obtained when eggs were left at a laboratory temperature.

TABLE IV.

Average Loss of Weight per Day of Eggs Incubated at Various Humidities.

No. of eggs weighed.	Average weight of eggs.	Temperature.	Humidity.	Average weight lost per day.
	gm.	*°C.*	*per cent*	*gm.*
4	58.0	39.6	93	0.044
52	57.7	38.2	90	0.066
12	54 3	39.2	80	0.155
12	58.0	39.2	77	0.180
31	60.6	38.2	65	0.242
10	58.8	38.5	52	0.345
10	59.0	21.7	38	0.220
16	57.1	38.2	23.5	0.533
12	59.8	39.2	20	0.610
4	61.2	39.2	20	0.660
16	60.8	40.6	16	0.640

There are factors of less importance. When eggs are isolated so that neither the sides of the drawer nor other eggs impinge upon them they lose more weight, and this occurs also when an electric fan blows air over their surfaces. Both arrangements serve to decrease the humidity in the immediate vicinity of the eggs and thus to favor evaporation.

In other experiments we have made certain that for our purposes no account need be taken of the slight differences in average daily weight loss which are occasionally found during the first 2 or 3 days of incubation. When the eggs are first put into the incubator they are cold and their water content is variable. Due to such factors the loss of weight which commences immediately may be less regular and different from the constant rate attained later.

. The divergence on the part of the fertile eggs from the straight line during the last 3 or 4 days of incubation (Fig. 3) may, however, be of some significance to this study. Its explanation may furnish a clue to metabolic changes of importance. Any hypothesis descriptive of the phenomenon must take into account the following experimental facts: (1) Whereas the unfertile eggs (◉) continue losing weight at a constant rate, the daily decrement for the fertile (o) eggs becomes greater. It cannot be due, as was first thought, to an unusually long exposure (about 1 hour) to a humidity of 23 per cent while some of the eggs were being weighed and opened, because the unfertile eggs (◉) which were used as controls, were subjected to the same conditions. (2) The extra loss of weight is slight, relative to the average daily decrement in eggs incubated at 23.5 per cent humidity, but in those kept at humidities of 65 and 90 per cent the increase in the daily loss during these last days of incubation is approximately equal to the previous average daily loss.

Three possible interpretations of the observed phenomenon present themselves: (1) that the expired carbon dioxide is greater by weight than the oxygen inhaled during this period, (2) that some other gaseous product is eliminated, and (3) that there is an increased evaporation of water. The first two possibilities apparently do not fit the facts. As embryos, of the same age, developed at humidities of 65 and 90 per cent, respectively, were approximately of equal weight regardless of the humidity, the increased decrement, which was *different* for each humidity, could not have been due to the loss of some metabolic product, such as carbon dioxide because the latter being·a function of some embryonic dimension would have been of the *same* magnitude under both conditions. Furthermore, if the loss of weight is due to the carbon dioxide output it must be the result of protein catabolism, since glucose is present in such small quantities that it may be neglected and when fat is burned the oxygen absorbed is greater by weight than the carbon dioxide lost. The urinary function in the chick has been said to start during the 2nd week and there is usually some fecal material in the amniotic cavity by the end of incubation. Even though no nitrogen is lost, there may be and probably is some protein catabolism, the products being eliminated in the usual forms, such as urea, urates, creatinine, and amino acids into the amniotic

fluid. Whether some of these nitrogen catabolites are reabsorbed is unknown. It seems that but little protein is burned during incubation, because in the metabolic experiments of Bohr and Hasselbalch (9) the respiratory quotient, during the latter half of incubation at any rate, was found to be about 0.710. Tangl's experiments (8) also point to the fact that practically all the energy involved is at the expense of fat.

In considering the effect of the gas exchange upon the weight of the egg, reference may be made to the estimations of Loewy which indicate that for every 100 cc. of CO_2 eliminated as a result of protein metabolism there is a loss of 0.0181 gm. of weight; whereas for every 100 cc. of CO_2 from fat there is a gain of 0.0056 gm. of weight (Table V). Even if one assumes that protein contributes as much as 20

TABLE V.

The Respiratory Exchange and the Change in Weight When Protein and Fat Are Oxidized (after Lusk[3]).

1 gm. of solid.	Respiratory gases.				Difference in weight per gm. of solid burned.	Difference in weight per 100 cc. CO_2 expired.
	Oxygen.		Carbon dioxide.			
	cc.	gm.	cc.	gm.	gm.	gm.
Protein.	966.3	1.380	773.9	1.520	−0.14	−0.0181
Fat.	2,019.3	2.885	1,427.3	2.805	+0.08	+0.0056

per cent of the CO_2 expired daily during the latter part of the incubation period, the loss from this source ($0.0181 \times 0.20 = 36.2 \times 10^{-4}$) would be more than neutralized by the gain in weight from fat metabolism ($0.0056 \times 0.80 = 44.8 \times 10^{-4}$). Much careful experimentation has been done by Hasselbalch (10) and others to discover whether ammonia or some other N-containing gas is eliminated by fertile eggs. It was generally believed that some small variable fraction of expired air was in this form, until the work of Krogh (11) and Tangl (8) which seemed to show that there was no loss of nitrogen during incubation. Our experiments are not intended to settle this point. The literature on the subject has led us to believe that its solution is of no particular

[3] Lusk, G., The elements of the science of nutrition, Philadelphia and London, 3rd edition, 1921, 62.

moment to our problem, since the amount of weight, if any, lost in such a form would appear to be practically negligible. Therefore, by a process of elimination we are led to the conclusion that the increased loss of weight during the latter part of incubation is due to an excessive elimination of water.

It appears consequently that changes taking place in fertile eggs result in a modification of the factors conditioning evaporation. The temperature of the living organism is higher than that of the surrounding atmosphere, and the amount of heat manufactured is roughly proportional to the mass of active tissue. Therefore, when the embryo has attained significant size, the heat radiating from its surfaces modifies temperature conditions in the whole egg. We have observed under standard conditions that a thermometer placed against the shell of a fertile egg gives a reading, the height of which above the surrounding temperature varies in general with the weight of the embryo. The change is not noticeable until near the end of incubation. As vapor tension is a function of temperature, it would seem on theoretical grounds that in consequence of the amount of heat produced by embryos of advanced age there should be an acceleration of evaporation, other factors being equal, during the last few days of incubation. Possibly the circulation of blood in the respiratory membrane which at this period covers the inner surface of the shell affords a more effective evaporating mechanism than the undifferentiated albumin. Finally, as will be shown later, the concentration of water in the egg rises during these last few days of incubation due to catabolic processes within the embryo so that for this reason the vapor tension and, consequently, the evaporation of water may be increasing. These suggestions are offered as explanations of the increased increment loss of weight during the last days of incubation. In the eggs incubated at very low humidities (23 per cent), however, there seem to be factors within the egg which limit evaporation.

In view of such considerations regarding the excessive evaporation of water at humidities between 65 and 90 per cent greater accuracy is obtained on the 17th, 18th, and 19th days of incubation by substituting for equation (4) the following

$$H = 7.5 \ (100 - h)t + 7.5 \ (100 - h) \ (t - 16) \tag{5}$$

where H = water lost by evaporation (mg.), h = humidity (per cent), and t = incubation age (days).

The equations which are now available express approximately the daily loss of weight of the whole egg (and thus the evaporation of water) as a function of the humidity, and the loss of weight of the shell (and thus the gain in weight of total solids within the egg) as a function of time. At any time during incubation by the use of one or more of these equations the original weight of the contents of any egg may be estimated, and thus an approximate value for the quantity of solid material *before* incubation may be obtained. By comparing the latter with the amount of solid material actually found *after* incubation it should be possible, despite the great variability of the eggs, to calculate within limits how much solid material is burned during incubation.

TABLE VI.

Analysis of Unincubated Eggs.

	No. of eggs analyzed.	Per cent of egg contents.	Per cent of total solids.	Gm. per 100 gm. H₂O.
Total solids............................	37	24.7 ±0.1	100	32.8
Fat.................................	11	11.1	45.0 ±0.8	14.7
Nitrogen............................	9	1.9	7.6 ±0.1	2.5
Protein (6.25 N).....................		11.7	47.5	15.5

The water content of the egg was obtained by subtracting from the weight of the whole egg the dried weight of the contents and the dried shell. Before drying the shell the albuminous material adhering to the inner surface of the shell membrane was washed off with distilled water and included with the rest of the egg contents. The egg substance was dried, (1) exposed to the air in an oven at 102°C.; (2) in a vacuated desiccator over H_2SO_4 at 60°C.; and (3) in a vacuated desiccator over phosphorous pentoxide at 102°C. The results obtained by the three methods were comparable, and as the first was the simplest it was thenceforth used exclusively.

The average concentration of water in thirty-seven eggs before incubation as they were received in the laboratory was 75.3 ± 0.1 per cent. The concentration of solids was therefore 24.7 ± 0.1 per cent (Table VI).

Let

W = weight of whole egg before incubation.

L_0 = weight of shell before incubation.

L_t = weight of shell after t days of incubation.

W_e = weight of embryo.

S_0 = weight of solids before incubation.

S_t = weight of solids after t days of incubation.

S = weight of solids burned.

Now since

$$L_0 = L_t + 0.01\ W_e \qquad (1)$$

and the solids represent 24.7 per cent of the egg contents, then

$$S_0 = 0.247\ (W - L_t - 0.01\ W_e)$$

and

$$S = 0.247\ (W - L_t - 0.01\ W_e) - S_t$$

The terms on the right side of the equation are all measurable.

Two sets of fertile eggs incubated at 90 and 65 per cent of humidity respectively were analyzed after incubation (Table VII). They are peculiarly irregular, but the averages seem to show a certain uniformity. As will be seen later there are reasons to believe that these are sufficiently accurate for our purposes.

When the humidity of the incubator air is changed, there is an accompanying change in the concentration of substances composing the yolk and albumin due to the evaporation of water of the eggs. Within rather wide limits such modifications have, however, no measurable effect upon the development and chemical differentiation of the embryo. The passage of substances into the embryo is not conditioned to any great extent by such external factors. As with many glands and other organs in the body, there appears to be evidence of selective absorption, so called, which to a large extent is independent of the concentration or total mass of the substance to be absorbed. The devices used to symbolize membrane equilibria in non-living colloidal systems are therefore not descriptive of the phenomena under observation; and one must resort in the present state of ignorance to certain specifically biological concepts. Haldane (12) believes, it seems, that the latter are axioms, characteristic of

TABLE VII.

Solids Oxidized during Incubation.

Humidity (per cent)	Incubation age (days)	Egg No.	W (gm.)	L_t (gm.)	Average ± standard error.*					
					W_e (gm.)	W_0 (gm.)	$S_0 + L$ (gm.)	S_t (gm.)	S (gm.)	$\dfrac{S_t}{H_t}$ (gm.)
90	17	4,7,8,9,19, 22,26,27.	58.93 ± 0.84	5.27 ± 0.23	20.35 ± 0.39	53.46 ± 0.67	13.41 ± 0.17	12.24 ± 0.21	1.16 ± 0.06	30.6 ± 0.2
	18	41,45.	51.98 ± 1.84	5.00 ± 0.20	22.15 ± 1.35	46.75 ± 1.67	11.77 ± 0.40	10.58 ± 0.76	1.19 ± 0.35	29.9 ± 1.3
	19	32,46,48, 54,53.	59.08 ± 1.88	5.62 ± 0.16	29.64 ± 0.67	53.15 ± 1.73	13.42 ± 0.43	11.63 ± 0.50	1.79 ± 0.22	28.8 ± 0.72
65	16	77†	58.49	5.22	14.45	53.12	13.26	12.46	0.80	34.1
	17	84,86,73.	60.77 ± 1.11	6.20 ± 0.23	17.48 ± 0.32	54.39 ± 0.68	13.60 ± 0.17	12.61 ± 0.10	0.99 ± 0.14	33.4 ± 0.51
	18	56,58.	57.74 ± 1.79	5.42 ± 0.10	20.78 ± 1.65	52.10 ± 1.68	13.07 ± 0.42	11.86 ± 0.78	1.20 ± 0.35	32.3 ± 1.5
	19	60,78,79, 90,76.	62.07 ± 2.12	5.75 ± 0.22	25.78 ± 1.12	56.06 ± 1.95	14.09 ± 0.54	12.55 ± 0.48	1.53 ± 0.23	32.5 ± 0.85

* Standard error = $\dfrac{\text{standard deviation}}{\sqrt{\text{No. of observations}}}$.

† Individual determination.

W = weight of the whole egg before incubation; L_t = weight of shell.

W_e = weight of embryo; $W_0 = W - L_t - 0.01$; W_s = weight of egg contents before incubation.

$S_0 = 0.247$; W_0 = weight of solids before incubation.

$L = 0.01$; W_e = weight of solids added to egg contents; $S_0 + L$ = solid content on the assumption that no solids are oxidized;

S_t = solid content as found; S = solid burned.

$\dfrac{S_t}{H_t}$ = solids in whole egg (gm. per 100 gm. of H₂O).

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and peculiar to living organisms and not to be resolved into chemical and physical terms. In pursuit of this general idea and in order to gain information of the phenomena underlying the movement of elements in and out of the embryo (absorption and elimination) it is desirable to know in approximate terms the changes in the concentration of the more important constituents of the whole egg during incubation.

It was necessary to know not only what substances are burned but also at what rates. In view of the literature on the subject the solids were analyzed for fat. The results may be seen in Table VIII. The fat content was determined by alcohol-ether followed by pure ether extraction in a Soxhlet apparatus. Before extraction and once during the latter part of the extraction process the dried substance was thoroughly ground in a mortar. The extraction process lasted about 20 hours. After extraction the flasks were dried to constant weight over paraffin in a vacuated desiccator. The term "fat" is henceforth used as synonymous with the extract thus obtained.

Since individual eggs show considerable variation our results are not very regular. The sensible maximum deviations in the content of total solid and fat before incubation (Table VI) were 1.6 per cent and 4.0 per cent, respectively; this amounts, then, to a possible error of $\pm$ 0.8 gm. in our calculations for S_0 (solid content before incubation (Table VII)) and approximately $\pm$ 1.3 gm. for F_0 (fat content before incubation (Table VIII)), since the latter estimation involves both errors. The maximum deviations from the mean in our values for S, the amount of solid burned during incubation, were well within these figures (Table VII), and therefore we were not led to seek further for an explanation of the apparently irregular variations. A comparison between the total amount of solid substance (28.362 gm.) and of fat (28.059 gm.) lost during incubation by twenty-two eggs shows ·a close correspondence (Table VIII). Approximately 98 per cent of the oxidized solid substance seems to be fat. This is corroborative of previous work, but may not be more accurate than $\pm$ 10 per cent. Tangl and von Mituch (8) found by the analysis of the contents of six fertile eggs after the incubation period that practically all the loss of solids could be accounted for by the oxidation of fat. Bohr and Hasselbalch (9) came to the same conclusion when they found that

TABLE VIII.

Fat Oxidized during Incubation.

Humid-ity.	Incu-bation age.	Egg No.	Average ± standard error.*					
			W_e	W_0	S_0	F_0	F_t	F
per cent	*days*		*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
90	17	4,8,9,19,22,26,27.	20.24 ± 0.43	53.57 ± 0.77	13.23 ± 0.19	5.95 ± 0.09	4.92 ± 0.12	1.04 ± 0.12
	18	41†	20.32	49.09	12.13	5.45	4.14	1.31
	19	32,46,48,54,53.	29.65 ± 0.67	53.15 ± 1.74	13.13 ± 0.41	5.90 ± 0.19	4.28 ± 0.36	1.63 ± 0.26
65	16	77†	14.46	53.12	13.12	5.90	5.13	0.77
	17	86,73.	17.65 ± 0.44	53.63 ± 0.41	13.24 ± 0.10	5.96 ± 0.05	4.95 ± 0.11	1.01 ± 0.06
	18	56,58	20.78 ± 1.65	52.10 ± 1.68	12.87 ± 0.41	5.79 ± 0.18	4.57 ± 0.55	1.22 ± 0.37
	19	60,78,79,76.	25.10 ± 1.17	54.60 ± 1.9	13.49 ± 0.47	6.07 ± 0.20	4.54 ± 0.21	1.53 ± 0.17

Total F.. = 28,059 gm

" S (Table VII) from same eggs (*i.e.* omitting Nos. 7, 45, 84, and 90)............. = 28,362 "

* Standard error = $\dfrac{\text{standard deviation}}{\sqrt{\text{No. of observations}}}$.

† Individual determination.

W_e = weight of embryo; W_0 weight of egg contents before incubation (from Table VII).

S_0 = 0.247; W_0 = weight of solid before incubation.

F_0 = 0.45; S_0 = weight of fat before incubation; F_t = weight of fat after t days of incubation.

F = fat burned.

the respiratory quotient for chicken embryos during the greater part of incubation was approximately 0.710. It must be kept in mind that the available figures show irregular variations, that the respiratory quotients for egg fats and proteins are not accurately known and that other transformations (carbohydrate formation from protein or fat) may take place within the embryo to vitiate conclusions from respiratory data. Nevertheless in view of the literature and our own confirmatory data it seems that *for our calculations* it is a justifiable assumption that fat only is oxidized during incubation.

There are certain reasons, as Tomita (13) and more recently Needham (14) have pointed out, for supposing that some glucose is metabolized before the 10th day of incubation. In the first place Bohr and Hasselbalch found a respiratory quotient of 0.89 as an average for the first 5 days of incubation; secondly the trace of glucose, which is known to be present at the start of incubation, is no longer to be found in the egg contents outside of the embryo after the 1st week of incubation (15, 16); and finally the curve for the concentration of lactic acid in the whole egg which was constructed by Tomita as a result of a large number of determinations, and is an index according to the author of glucose metabolism, rises at the start of incubation, reaches its maximum on about the 4th day, and then declines.

It is uncertain when the end-products of nitrogen metabolism first appear in the allantoic sac; but if experiments were to prove that there was a procession of glucose, protein, and fat as sources of energy the findings might be correlated to the change with age in the value of the respiratory quotient as portrayed in Needham's graphical representations of Bohr and Hasselbalch's figures. Later it may be possible to determine the truth of this hypothesis and to estimate how much carbohydrate and protein metabolism occurs. The embryo's mass is so insignificant in the beginning of incubation that during the first $9\frac{1}{2}$ days (one-half of the period under observation) only one-seventeenth of the amount of solid substance eventually to be burned is lost. There would probably be an error of no more than 5 per cent if the catabolism of the first half of incubation were calculated on the basis of fat, instead of carbohydrate and glucose.

Assuming then that only fat is oxidized, determinations were made of the rate of fat catabolism at each incubation age. There were

before us the tabulations of total oxidized solid (Table VII) for the 16th to the 19th day inclusive, but these were not sufficient to extrapolate earlier values. There were also at hand the careful researches of Bohr and Hasselbalch (9, 17) on the metabolism of chicken embryos; but in their experiments the breed of the hen laying the egg was not recorded, the conditions of incubation were not fixed, the chicken weights for some ages were quite different from our own, the weights of the embryos actually used in the metabolic tests were not determined, and finally inspection revealed unusually large variations in the results. It was therefore considered necessary to determine for ourselves the carbon dioxide expiratory rate throughout incubation.

Carbon Dioxide Production.

The apparatus used was similar to the conventional pattern (Fig. 5). Air was drawn by suction at a constant rate through soda-lime and water vapor into a glass bottle containing the egg, and then successively through sulfuric acid (tube G), soda-lime (tube H), and sulfuric acid (tube J). The difference between the combined weights of tube H and tube J, before and after a certain interval of time, is the weight of carbon dioxide eliminated by the egg in that time.

The following experiments were made and repeated in order to test the efficiency of the apparatus:

(1) Barium hydroxide in tubes through which CO_2-free air had been passed were placed in the circuit after vessel C and tube J. Absence of turbidity, or a precipitate, indicated that the CO_2 was being properly absorbed.

(2) A second sulfuric acid tube was put into the circuit after tube G. No change in its weight after a long period of circulation indicated that tube G was absorbing all the water.

(3) Air was circulated for several hours through the empty apparatus. There was no change in the combined weight of tubes H and J.

The rate of flow of air which was nearly saturated with water in all experiments was constant at 3 liters per hour, so that in the larger embryos only did the surrounding atmosphere approach a concentration of CO_2 as great as 0.5 per cent. In some of Hasselbalch's experiments the concentration rose above 0.5 per cent without any noticeable change in the metabolic rate, according to the authors, so that we were led to consider this a factor of little significance. It was found

that within wide limits a change in the humidity during the short time that the test lasted did not affect the rate of CO_2 elimination.

Bohr and Hasselbalch laid considerable emphasis upon the elimination of CO_2 by the shell. In these experiments it was assumed that after carbonic acid equilibrium had been fixed between the egg and the surrounding atmosphere there would be no measurable interchange in either direction, and that this condition could be achieved by allowing for a preliminary period of circulation before commencing the experiment. No test was commenced until air had flowed through the apparatus at the same constant rate for at least half an hour in
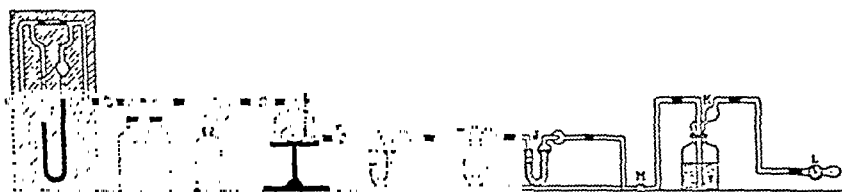
FIG. 5. Diagram of apparatus set up on a table in a constant temperature room to measure the carbon dioxide production of chicken embryos.

A = flow-meter; B = bottle of moist soda-lime; C = bottle with a small quantity of barium hydroxide solution at the bottom for the purpose of moistening the air passing over it and of giving evidence, by the formation of a thin superficial film, of the presence of any CO_2 in the air; D = egg resting in a glass bottle with two outlets and a ground glass stopper through which passes E; E = thermometer, the bulb of which lies against the shell; F = two-way stop-cock; G = pumice and sulfuric acid tube; H = moist soda-lime tube; J = pumice and sulfuric acid tube; M = two-way stop-cock; K = trap; L = dial on a needle valve to indicate flow of air into suction tube.

the older embryos and for an hour in the embryos less than 10 days old. In a number of the experiments values were obtained for the CO_2 output of single embryos over short successive periods. The lack of a regular decline or increase in their metabolism showed that a plateau or a relatively constant rate is attained after half an hour.

Mr. J. B. S. Haldane has called my attention to the fact that as the egg white has an alkaline reaction during the early stages and is said later to become acid (18), the change in pH may be due in part to the retention of CO_2 formed in metabolism. The figures for CO_2 elimination, particularly for the younger embryos may accordingly be too

low. Moreover, as mentioned above, the influence of the carbonates from the shell is at present undetermined. As they enter into solution there may be a retention of CO_2 and as they become reprecipitated in the bone, there may be an equivalent liberation of the gas. If these

TABLE IX.

Carbon Dioxide Production of Chicken Embryos.

Experi-ment No.	Egg weight.	Age.	Embryo.			Tempera-ture.	Dura-tion of test.	CO_2 per 24 hrs.	CO_2 per 24 hrs. per gm.
			Weight.	Log weight.	Sex.				
	gm.	days	mg.			°C.	hrs.	cc.	cc.
1	57.8	6	330	2.52	M.	38.5	5	9.5	28.6
2	59.4	6	526	2.72		38.0	6	15.7	29.8
3	60.5	8	815	2.91		38.8	6	25.2	30.9
4		7	832	2.92		38.7	3	25.1	30.1
5	57.0	8	1,213	3.08	"	38.9	4	31.5	26.1
6	58.3	9	1,542	3.19		38.8	4	46.3	30.0
7	58.9	9	1,726	3.24	"	38.7	6	50.9	29.5
8	59.9	10	2,534	3.40		38.8	5	70.3	27.8
9	59.5	11	3,494	3.54	F.	38.7	5	94.5	27.1
10	60.2	11	3,650	3.56	M.	38.7	1	101.8	27.8
11	58.3	12	3,812	3.58	F.	38.9	1	106.9	28.0
12		12	5,411	3.73	M.	38.8	2	150.3	27.8
13	57.1	13	8,629	3.94	F.	39.0	1½	213.3	24.7
14	57.1	14	10,618	4.02		38.8	1	259.3	24.7
15	58.0	14	11,562	4.06		37.8	1	261.7	22.6
16	59.1	14	12,113	4.08	"		1	230.3	19.0
17	56.4	15	14,862	4.17	"	39.1	1½	322.4	21.7
18	56.6	16	15,105	4.18	"	39.0	1½	320.0	21.2
19	58.4	16	18,229	4.26	M.	39.0	1	363.6	19.9
20	58.7	18	22,314	4.35	F.	38.7	1½	373.3	16.7
21	56.3	17	22,745	4.36	"	38.6	1	387.8	17.0
22	63.6	18	25,850	4.41	"	38.9	5½	357.0	13.8
23		16	15,826	4.20	M.	38.9	1	326.0	20.6
24		17	18,181	4.26	"	38.8	1	348.0	19.1
25		18	23,968	4.38	F.	38.8	1	358.0	16.9
26		19	25,218	4.40	"	38.7	1	368.0	14.6

two processes are not occurring simultaneously there will be errors in the figures for metabolism. Some of the more evident sources of error such as these will be investigated at a later date, but in the present study they have been neglected.

The results of our experiments have been tabulated (Table IX) and

the CO_2 production per 24 hours, equated against the logarithm of the embryonic weight (Fig. 6). Through the points on the chart a smooth curve may be drawn and then may be read off on the curve the values (Column 2, Table X) which correspond to the logarithms of the weights reported elsewhere as the averages for their respective ages (Column 1, Table X). This indirect method of arriving at the CO_2 production rate in terms of incubation age is more accurate, since
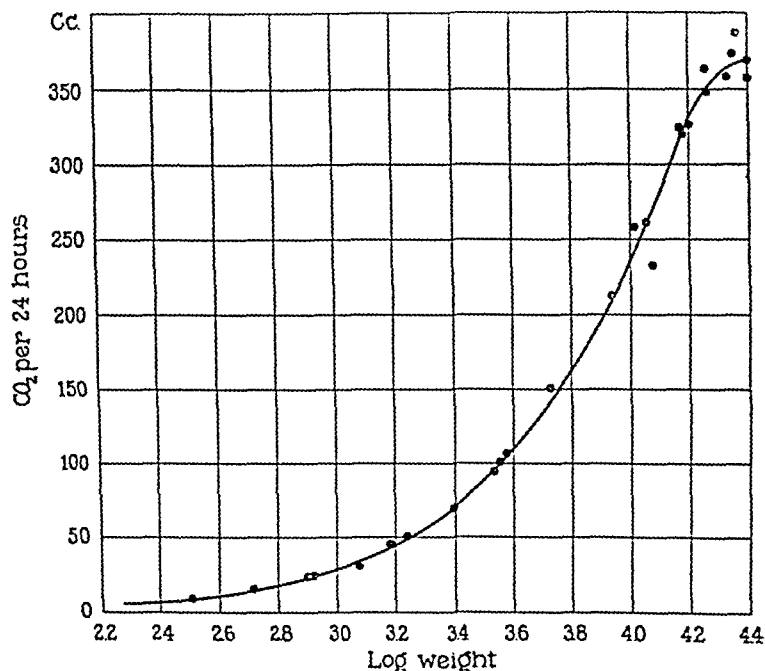
FIG. 6. The CO_2 production per 24 hours equated against the logarithm of the wet weight of the embryo. The graph to describe theoretically the locus of the points was drawn by inspection (Table IX).

the correlation of catabolism with embryonic weight is closer than with age. The average embryonic weight at each age has been fixed with relative certainty by several hundred weighings. It is therefore justifiable to draw the curve expressing the rate of CO_2 production in terms of embryonic age (Fig. 7). By measuring with a planimeter the area under the graph for each day, the integrals may be obtained which give the total CO_2 elimination since the beginning of incubation

as a function of time (Column 3, Table X). Assuming that the CO_2 is derived from fat catabolism the values given in Table V may be used for calculating total solids burned since the beginning of incubation (Column 4, Table X). These may then be compared to the figures obtained from the chemical analyses (Columns 5 and 6, Table X). There can be little question but that the correspondence is satisfactory (Fig. 8).

TABLE X.

Catabolism as a Function of Embryonic Age and Weight.

Age.	(1) W_e Embryo weight.	(2) CO_2 per 24 hrs.	(3) Total CO_2.	(4) F or S Total solid (calculated).	(5) Total solid (determined).	(6) Total fat (determined).
days	*mg.*	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*
4			2.0	0.001		
5	221	6	6.0	0.004		
6	423	12	14.4	0.010		
7	735	22	30.4	0.021		
8	1,189	35	58.4	0.041		
9	1,879	54	104.0	0.073		
10	2,661	74	168.0	0.118		
11	3,750	102	254.0	0.178		
12	5,105	132	368.0	0.258		
13	6,839	171	518.0	0.363		
14	8,974	215	706.0	0.495		
15	11,460	262	940.0	0.659		
16	14,390	315	1,224.0	0.857	0.800	0.773
17	17,950	350	1,560.0	1.093	1.081	1.023
18	22,030	366	1,922.0	1.346	1.199	1.267
19	26,670	368	2,290.0	1.605	1.664	1.577

In Column 1 are the embryonic weights at different ages as obtained from the formula $W_e = \dfrac{t^{3 \cdot 8}}{1.496}$.

In Column 2 are the values for the rate of CO_2 production as read from the curve in Fig. 6.

In Column 3 are given the figures for the total amount of CO_2 produced since the beginning of incubation as obtained by summing the areas under the graph in Fig. 7.

The values in Column 4 were obtained by assuming that only fat substances were oxidized and that 1 cc. of CO_2 is equivalent to approximately 0.0007 gm. fat.

The figures in Columns 5 and 6 are derived from Tables VII and VIII respectively.

.There are now sufficient data at hand to calculate the concentration of solid substance of an egg of average size throughout incubation under any conditions of humidity. The results are shown in Table XI, and below the table are given the values and empirical equations which were used in the calculations.
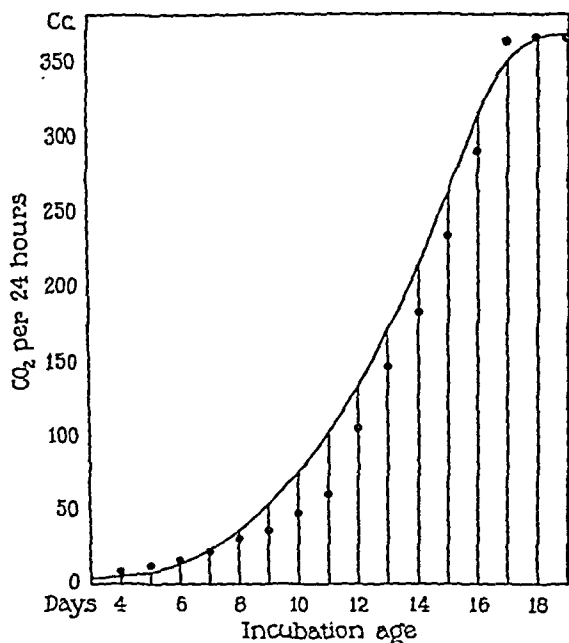
FIG. 7. A graph drawn to show the rate of CO_2 production per 24 hours as a function of age (Column 2, Table X). The perpendicular lines define areas which represent the total CO_2 production for each 24 hour period. The summation of these will give the CO_2 production since the beginning of incubation (Column 3, Table X). The black circles are the points given by Bohr and Hasselbalch.

The curves in Fig. 9 represent the concentrations of solid substance as calculated during the entire incubation period for humidities of 67.5 per cent and 91 per cent.[4] It is seen that the correspondence between the actual values as represented by circles and the theoretical curves is close, an observation which supports our assumption that the various steps whereby these values were obtained have empirical justification.

[4] Corrected from 65 and 90 per cent respectively according to Fig. 4.

In the eggs incubated at 90 per cent humidity the embryos were in general somewhat larger, but it was quite common to find mold colonies adherent to the inner surface of the shell.   Due to the fact that the humidity under a hen is said to be about 60 per cent and that our eggs kept under these conditions (65 per cent) gave the highest percentage of live embryos, it was decided to maintain the humidity
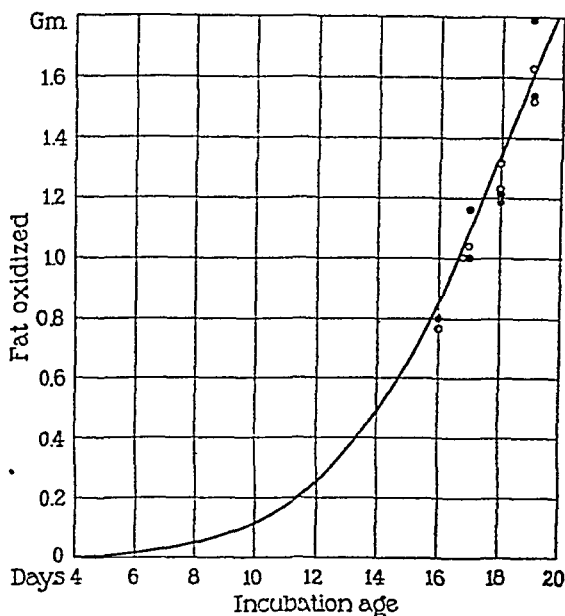
FIG. 8. A curve to show the total amount of solid (or fat) oxidized during incubation when the assumption is made that fat only is burned.   The graph may be compared to the black circles (●) which represent total solids, and the white circles (o) which represent fat as actually determined (Tables VII and VIII).

constantly between 65 and 70 per cent (average 67.5 per cent) and the temperature at 38.8 ± 0.4°C.

With these data at hand one can construct a theoretical table, which will give the content of total solids, fat, and nitrogen for each day of incubation under those conditions which have been arbitrarily selected as a standard (Table XII).

TABLE XI.

Concentration of Solids in the Whole Egg during Incubation.

Age.	W_e	L	S	S_t	Humidity 91 per cent.				Humidity 67.5 per cent.			
					H	W_t	H_t	$\dfrac{S_t}{H_t}$	H	W_t	H_t	$\dfrac{S_t}{H_t}$
days	*mg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*	
0				12.86	0	52.10	39.24	32.8	0	52.10	39.24	32.8
1				12.86	0.07	52.03	39.17	32.8	0.24	51.84	38.98	33.0
2				12.86	0.13	51.97	39.11	32.9	0.48	51.62	38.76	33.2
3				12.86	0.20	51.90	39.04	32.9	0.73	51.37	38.51	33.4
4				12.86	0.26	51.84	38.98	33.0	0.97	51.13	38.27	33.6
5	221			12.86	0.33	51.77	38.91	33.0	1.21	50.89	38.03	33.8
6	423		0.01	12.85	0.40	51.70	38.85	33.1	1.45	50.65	37.80	34.0
7	735	0.01	0.02	12.85	0.46	51.65	38.80	33.1	1.69	50.42	37.57	34.2
8	1,189	0.01	0.04	12.83	0.53	51.58	38.75	33.1	1.94	50.17	37.34	34.4
9	1,879	0.02	0.07	12.81	0.59	51.53	38.72	33.1	2.18	49.94	37.13	34.5
10	2,661	0.03	0.12	12.77	0.66	51.47	38.70	33.0	2.42	49.71	36.94	34.6
11	3,750	0.04	0.18	12.72	0.73	51.41	38.69	32.9	2.66	49.48	36.76	34.6
12	5,105	0.05	0.26	12.65	0.79	51.36	38.71	32.7	2.90	49.25	36.60	34.6
13	6,839	0.07	0.36	12.57	0.86	51.31	38.74	32.5	3.14	48.06	36.46	34.5
14	8,974	0.09	0.49	12.46	0.92	51.27	38.81	32.1	3.39	48.80	36.34	34.3
15	11,460	0.11	0.66	12.31	0.99	51.22	38.91	31.6	3.63	48.58	36.27	34.0
16	14,390	0.14	0.86	12.14	1.06	51.18	38.04	31.1	3.87	48.37	36.23	33.5
17	17,950	0.18	1.09	11.95	1.19	50.09	39.14	30.5	4.36	47.92	35.97	33.2
18	22,030	0.22	1.35	11.73	1.32	50.00	39.27	29.9	4.84	47.48	35.75	33.8
19	26,670	0.27	1.60	11.53	1.45	50.92	39.39	29.3	5.32	46.05	35.52	32.5

W = 57.8 = weight of whole egg (average 500 + eggs) before incubation.

L_0 = 5.7 = weight of shell (9.81 $\doteqdot$ 0.11 per cent) before incubation.

W_0 = 52.1 = weight of egg contents before incubation.

W_t = weight of egg contents after t days of incubation = $W_0 - H$.

H_0 = 39.24 = weight of water (75.3 per cent) before incubation.

H_t = weight of water after t days of incubation = $W_t - S_t$.

S_0 = 12.86 = weight of solids (24.7 per cent) before incubation.

S_t = weight of solids after t days of incubation = $S_0 - S + L$.

S = weight of solids burned (*cf.* Column 4, Table X).

$W_e = \dfrac{t^{3.8}}{1.496}$ = weight of embryo (mg.) (*cf.* Column 1, Table X).

$L = 0.01\ W_e = \dfrac{t^{3.8}}{0.1496}$ = weight of solids added to the egg contents from the shell (gm.).

H = 0.0075 (100 − h) t = water lost by evaporation (0 − 16 days).

H = 0.0075 (100 − h) t + 0.0075 (100 − h) (t − 16) = water lost by evaporation (17 − 19 days).

$\dfrac{S_t}{H_t}$ = solids (gm. per 100 gm. H_2O) after t days of incubation.

TABLE XII.

Solid, Fat, Nitrogen, and Water in Egg Contents during Incubation under Standard Conditions.

Age.	(1) Solids $= S_t$	(2) Water $= H_t$	(3) Fat $= F_t$	(4) Nitrogen$=N_t$	(5) Per cent $N =$ $\dfrac{N_t}{S_t}$	(6) C_t
days	gm.	gm.	gm.	gm.	per cent	
0	12.86	39.24	5.79	0.974	7.57	1.00
1	12.86	38.98	5.79	0.974	7.57	1.00
2	12.86	38.76	5.79	0.974	7.57	1.00
3	12.86	38.51	5.79	0.974	7.57	1.00
4	12.86	38.27	5.79	0.974	7.57	1.00
5	12.86	38.03	5.79	0.974	7.57	1.00
6	12.85	37.80	5.78	0.974	7.58	1.00
7	12.85	37.57	5.77	0.974	7.58	1.00
8	12.83	37.34	5.75	0.974	7.59	1.00
9	12.81	37.13	5.72	0.974	7.60	1.00
10	12.77	36.94	5.67	0.974	7.62	1.01
11	12.72	36.76	5.61	0.974	7.66	1.01
12	12.65	36.60	5.53	0.974	7.70	1.02
13	12.57	36.46	5.43	0.974	7.75	1.02
14	12.46	36.24	5.30	0.974	7.81	1.03
15	12.31	36.27	5.13	0.974	7.91	1.04
16	12.14	36.23	4.93	0.974	8.02	1.06
17	11.95	35.97	4.70	0.974	8.15	1.08
18	11.73	35.75	4.44	0.974	8.30	1.10
19	11.53	35.52	4.19	0.974	8.45	1.12

Columns 1 and 2 from Table XI.

Column 3, ($F_0 = 0.45\ S_0 = 5.79$ gm. (Table VI); $F_t = 5.79 - S$ (Column 4, Table X). The initial concentrations for fat and nitrogen are derived from Table VI.

Column 6, $C_t =$ the constant at t days, by which the initial preincubation concentration of any substance (if expressed as per cent of dry solid) must be multiplied to find the normal curve, which may be used as a base line for comparison with the values actually obtained. The reason for this is that due to the continuous loss of oxidizable solid during incubation the concentration of any substance, if unaffected during incubation, will nevertheless appear to rise, since the total amount of solid substance is falling.
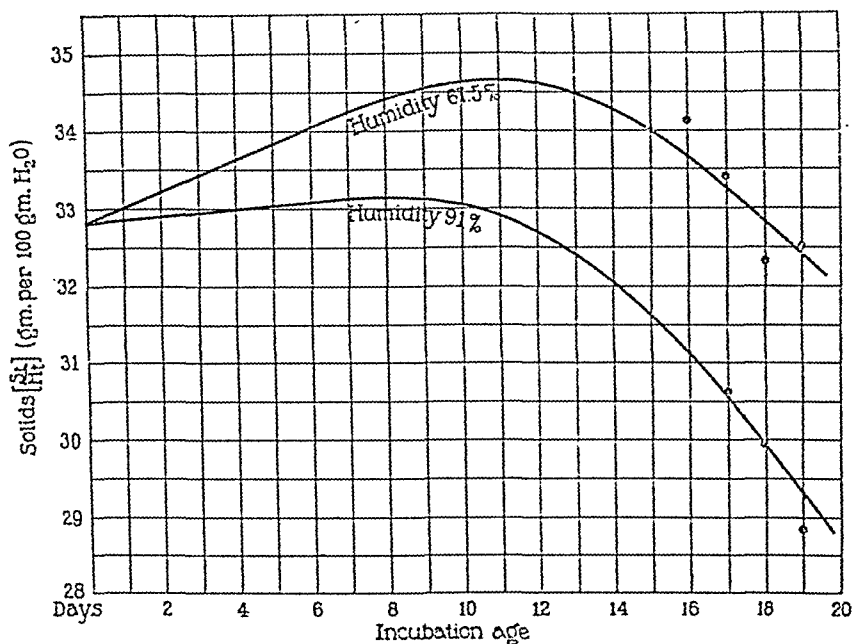
FIG. 9. Theoretical curves to indicate the changing concentration of solids (gm. per 100 gm. H₂O) in the whole egg during the incubation period at humidities of 67.5 and 91 per cent (Table XI). The black circles show the values actually obtained (Table VII) in eggs incubated at these humidites (corrected from 65 and 90 per cent respectively, according to the graph (Fig. 4).

DISCUSSION AND SUMMARY.

As this paper goes to press a complete review of the chemistry of the fertile egg will be appearing (19). The author, Mr. J. Needham, was kind enough to allow me to inspect his manuscript and thus avail myself of the comprehensive bibliography and discussion. It is surprising that no biochemists have estimated the changing water content of the egg during incubation. Many of the analyses reported in Needham's review were expressed in per cent of total weight or per cent of dry solid, and consequently are of questionable value, since these latter functions are themselves changing; the former due to water evaporation and the latter through the addition of shell constituents and the burning of oxidizable organic compounds. Moreover, there has been no statistical treatment of the results, and the reliability of

the average figures obtained has consequently been difficult to estimate.

Tangl's work, quoted throughout this paper, except for its lack of statistical treatment is more enlightening. However, his concept of the so called "Energy of Embryogenesis" which he propounds, seems to me misleading and unwarranted. What Tangl measured was the amount and the caloric value of the solid material burned and thus the quantity of energy lost during the embryonic period. The latter is equivalent to the usual measurements of catabolism. In the case of the embryo it is not basal metabolism which is being estimated, since the conditions are not basal. The embryo is absorbing and assimilating nutriment all the while at a relatively rapid rate.

The calorific value of the oxidized solid, which is in truth the amount of energy lost during a certain chosen interval, in Tangl's judgment stands for the energy of embryogenesis; *i.e.*, the energy of development (growth + differentiation). We believe that this conception is erroneous. The two processes, anabolism and catabolism, occur together and undoubtedly have some relationship, but surely one is not a measure of the other.

In a starving animal, and so probably in a starving embryo, there is a considerable amount of so called basal metabolism. Thus if the "Embryogenetic Energy" were measured under these conditions a figure would be obtained for which there was no growth to correspond, or in other words there would be a value for something which did not exist.

It will be seen in our later communications that the changes with age of metabolic rate and growth rate do not coincide. The amount of catabolism under certain circumstances does not accelerate growth or anabolism, but seems rather to be a limiting factor. It is as if when the absorbed energy were constant an increase of catabolism would make inroads upon the amount of energy which otherwise would remain for storage (growth).

If, as Pembrey's (20) experiments would tend to show, there is an increase of metabolism in the oldest embryos when the outside temperature is lowered, one would find at the end of incubation in such cases that there was a *greater* amount of so called "Energy of Development" but *smaller* embryo. It seems that the potential energy

amassed as growth comes from that remaining after the needs of the body have been satisfied.

The results of the experiments described in this paper have formed the basis for judgment in the selection of suitable standard conditions for the incubation of hen's eggs. Standardization was necessary so that in future experiments the more important environmental factors might be kept uniform within a certain appropriate range and therefore not be held accountable for deviations observed in the embryos.

Henceforth in this series of papers the term "standard incubation conditions" will signify that (1) the temperature was constantly at 38.8 $\pm$ 0.4°C., (2) the humidity at 67.5 $\pm$ 2.5 per cent, (3) there was a continuous flow of warm air into the incubator to provide the necessary circulation, and (4) the eggs were rolled once a day within the constant temperature room.

The incubator, a double-walled copper cabinet, stands in a constant temperature room, the fluctuations of which are $\pm$ 1.0°C. The space between the walls of the incubator is filled with water which serves as a buffer to outer variations.

It might be repeated that all the eggs are from White Leghorn hens, are incubated 2 days after laying, and that they are kept cold during the interval necessary for transportation.

With the figures from our chemical analyses and metabolic rate experiments, it was possible to calculate values for the concentration of total solids, fat, and nitrogen throughout the incubation period. These data were necessary as a general chemical background for further work. The results of the calculations are obviously rough. Because of the great variability of the eggs a satisfactory degree of accuracy could not have been attained without a very large number of analyses supplemented by complete statistical treatment. The necessity for such a comprehensive study was not evident, and it is our belief that the approximations reached in this paper are sufficiently close to serve our present purposes.

The chief facts that have been ascertained in this investigation are

(1) Loss of water by the egg during incubation is a function of the atmospheric humidity in its immediate environment. More rapid circulation of air lowers the humidity around the egg and thus increases

evaporation.   Other facts influencing evaporation are (a) atmospheric ·temperature, (b) thickness and surface area of the shell, and (c) conditions within the egg, the most important of which, it is suggested, is the amount of heat produced by the embryo.   The latter factor, in turn, depends upon its size and age, and a significant change does not become apparent until the last 3 or 4 days of incubation, that is to say, when the embryo is of sufficient mass to exert a measurable force.

(2) The surface area of the eggs in sq. cm. may be approximately represented by the formula $S = K W^{\frac{2}{3}}$, where $K = 5.07 \pm 0.10$, and W = the weight of the whole egg in gm.

(3) There is a loss of weight by the shell during incubation.   This is most noticeable near the end of the cycle, when the loss seems to parallel in general the weight of the embryo.

(4) There is also a loss of solid matter during incubation.   Chemical analyses indicate that about 98 per cent of the material oxidized is fat. This conclusion is corroborative of previous work by Hasselbalch, Hasselbalch and Bohr, and Tangl.

(5) Carbon dioxide may be measured with relative   accuracy. When it is assumed that it is derived from the oxidation of fat, satisfactory corroboration of the chemical analyses is obtained.

These experiments have furnished the data from which the values have been calculated for total solids, fats, and protein in the whole egg throughout incubation.   The figures may be used later for comparison with the concentration of these substances within the embryo.

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PHYSIOLOGICAL ONTOGENY.

A. Chicken Embryos.

III. Weight and Growth Rate as Functions of Age.

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Growth and division of cells represent one of the characteristic signs of life. In an analysis of the phenomenon of ontogenesis the general growth rate as estimated by the increase in mass must be included as one of its most striking and significant features. The present investigation concerns itself with the weight and growth rate of chicken embryos as functions of age. The source of the eggs and the management of incubation have been described in a previous paper (1).

Method.

Fertile eggs of from 5 to 19 days of incubation age were opened. After cutting the membranes the embryos were removed and separated at the distal end of the yolk stalk. Various methods were used in an attempt to prevent loss of blood from the vessels or retention of an undue amount of amniotic moisture on the skin. Laying the embryos momentarily on a piece of filter paper or on a smooth towel, or passing a current of air over their surfaces was tried. Occasionally the vessels were clamped. We were never confident of the accuracy of certain points in our technique in weighing the smaller embryos, such for instance as whether the water adhering to the skin compensated for the frequent loss of a drop or two of blood. In the older embryos only did we feel assured that the errors incurred from these sources were minimal. To include the amniotic and chorionic membranes, which would have made an appreciable difference in the weight was

deemed desirable from the theoretical consideration that these structures are composed of living cells, presumably sharing in the general metabolic activity of the body. To do this, however, without the loss of blood and without including other non-functioning parts and adherent water was found to be impractical. In this and subsequent researches then we have concerned ourselves only with the embryo itself, assuming that the error incurred by neglecting the membranes was either constant throughout incubation or negligible. In consequence, although the factors analysed, such as growth rate, metabolism, and chemical constitution, may be compared one with another they may have only an approximate general validity due to this omission. In some of the 19 day old embryos the yolk sac, which could not properly be considered a part of the living organism, had been taken up into the abdominal cavity, and so in such cases it was necessary to squeeze it out gently and separate it before weighing the chick.

The embryos were weighed in weighing bottles. With the younger chicks, when certain chemical analyses were to be made, a number of embryos were placed in the same bottle so that the variations of the individual weights for these ages could not be obtained. They were probably considerable.

Most of the embryos included in this particular investigation developed in a Lo-Glo incubator, that is to say before conditions had been standardized as outlined in the preceding communication. In this incubator the temperature conditions were not perfectly uniform, so that some eggs probably developed faster than others due to their position in the drawers. No provision was made for this discrepancy except that the eggs were rolled each day, the drawers frequently changed, and the weighings continued over an 8 months period so that the average value for any one age does not represent a particular group of embryos subjected to particular conditions. After the completion of this series, when a constant temperature room for our experiments had been constructed, we were able to make a number of weighings under standard temperature and humidity conditions for comparison.

The sex was not determined. The results, therefore, represent a mean average of the weights of White Leghorn embryos varying from

5 to 19 days of age and incubated at a temperature of about 39°C., and a humidity of about 70 per cent. It was hoped that with a rather large number of weighings the chances for the equalization of factors causing deviations from the mean, namely, of temperature, humidity, sex, and technical errors would be optimal.

TABLE I.

Weight, Growth Rate, and Acceleration of Growth of Chicken Embryos.

Age.	Log age.	No. of embryos.	Mean wet weight and probable error.*	Log wet weight.	Log W = 3.6 Log t − 0.175		Percentage growth rate. $\dfrac{dw}{dt}$ $\dfrac{}{W} = \dfrac{3.6}{t}$	Acceleration. $\dfrac{d}{dt}\left[\dfrac{dw}{dt}\dfrac{}{W}\right] = -\dfrac{3.6}{t^2}$
					Wet weight from formula.	Log wet weight from formula.		
days			*mg.*		*mg.*			
5	0.699	200	206	2.314	221	2.345	0.720	−0.1440
6	0.778	45	424	2.627	423	2.626	0.600	−0.1000
7	0.845	91	730	2.863	735	2.866	0.514	−0.0735
8	0.903	42	1,200 ± 19	3.079	1,189	3.075	0.450	−0.0562
9	0.954	48	1,916 ± 163	3.282	1,817	3.259	0.400	−0.0445
10	1.00	48	2,614 ± 23	3.417	2,661	3.425	0.360	−0.0360
11	1.041	27	3,738 ± 62	3.572	3,750	3.574	0.327	−0.0298
12	1.079	15	5,010 ± 116	3.700	5,105	3.708	0.300	−0.0250
13	1.114	35	7,239 ± 120	3.859	6,835	3.835	0.277	−0.0213
14	1.146	15	9,484 ± 220	3.977	8,974	3.953	0.257	−0.0184
15	1.176	12	11,734 ± 322	4.069	11,460	4.059	0.240	−0.0160
16	1.204	10	14,343 ± 242	4.156	14,390	4.158	0.225	−0.0141
17	1.230	35	18,364 ± 215	4.264	17,950	4.254	0.212	−0.0125
18	1.255	29	20,800 ± 334	4.318	22,030	4.343	0.200	−0.0111
19	1.278	30	26,341 ± 411	4.421	26,670	4.426	0.190	−0.0100

*This is the usual probable error of the biometrician; *i.e.*,

$$0.6745 \frac{\text{standard deviation}}{\sqrt{\text{number of observations}}}.$$

RESULTS.

The results have been summarized in Table I. Curves of the average weights (Fig. 1) and of their logarithms (Fig. 2) have been plotted as functions of age. The latter, which is comparable to percentage weight increments, shows by its slope that the greatest relative changes in weight occur in the early days. The percentage increase in mass rather than the actual increments of weight is taken

as the rate of growth because this aspect is considered biologically more significant.　Either definition is permissible, but from a functional standpoint it would seem that the object of interest is the growth and divisional rate per unit mass.　When the weight increments are taken as the basis of growth rate, the quantity of tissue taking part in the reaction is left out of consideration.　For instance, if an embryo
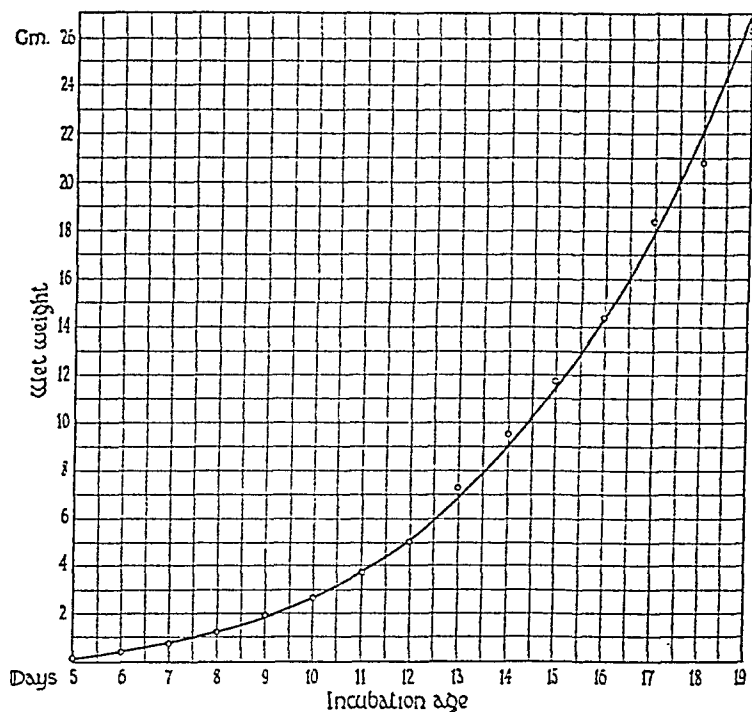
FIG. 1. To show the average wet weight of chicken embryos as a function of age (Table I).

weighing approximately 2.5 gm. is found to have gained 1.0 gm. in 24 hours its growth rate, using increments as the criterion, would be considered equal to that of a chicken weighing, let us say, 50.0 gm. which also gained 1.0 gm. in the same period.　From a physiological standpoint this conception of the rate of growth is misleading, and does not allow for a comparison between the same organism at different ages or organisms of different species at the same age.　Henceforth
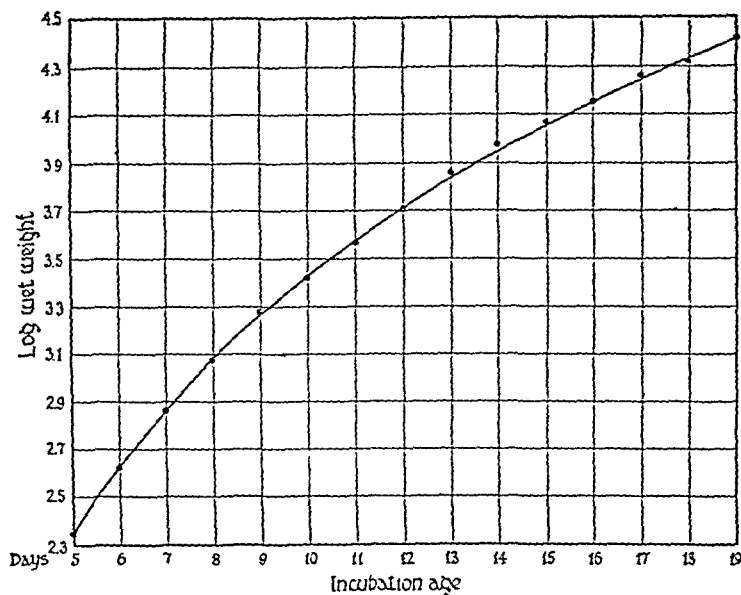
FIG. 2. The logarithm of the average wet weight equated against the age of the embryo (Table I).

$$\log w = 1.6 \log t - 0.175$$
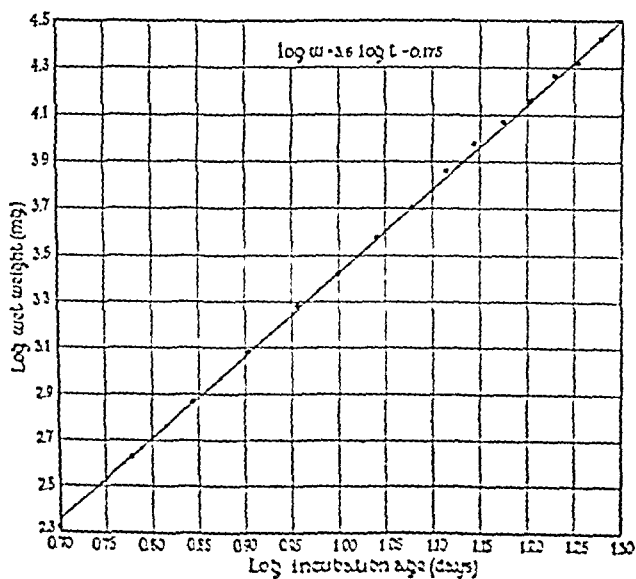
FIG. 3. The logarithm of the average weight expressed as a function of the logarithm of the incubation age in days (Table I).

when the term growth rate is used, we shall mean the percentage rate of increase in mass; *i.e.*, velocity $= v = \dfrac{\dfrac{dw}{dt}}{W} = \dfrac{d}{dt}\left[\dfrac{dw}{W}\right] = \dfrac{1}{W}\cdot\dfrac{dw}{dt}$.

It was found that when log W was equated against log t the points approximated a straight line (Fig. 3). The formula log W = 3.6
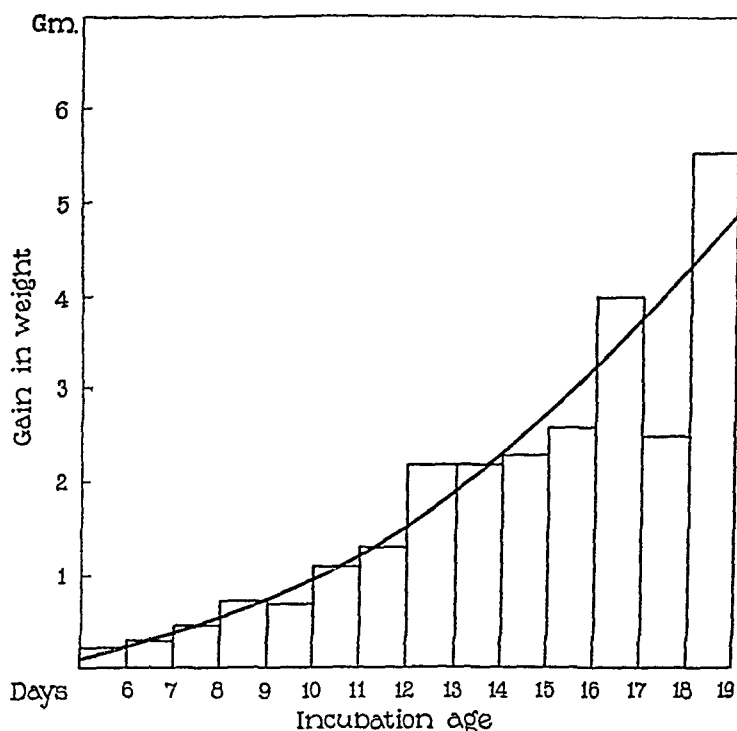
FIG. 4. The increments of growth for each day of incubation. The curve is the theoretical expression for the increments as derived from the formula.

$$W = 0.668t^{3.6}$$

log t — 0.175 was obtained graphically. By its use the graphs in all the figures were drawn. These figures are included in the table (Table I) so as to allow for comparison with the average findings. We do not attach theoretical significance to the fact that the weights can be expressed by such a simple equation. If certain intervals of growth are chosen, and particularly if these are sufficiently short,

it is not difficult to find simple equations to fit weight figures. Various mathematical expressions have recently been suggested to conceptualize the growth process. These may be useful devices but they do not furnish, in our judgment, sufficient evidence to establish the nature of the "master reaction" of growth (2). The embryonic period may be considered as one, or the major part of one, of the three chief cycles, or periods of accelerated growth, in the life span. The rate slows asymptotically as the chick approaches an equilibrium with its immediate environment before hatching. When the increments of
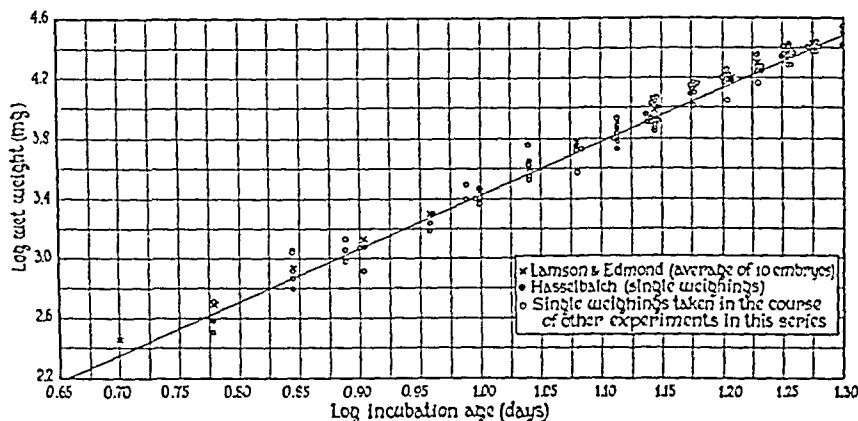
FIG. 5. The graph obtained from the formula $\log W = 3.6 \log t - 0.175$ compared to the average results obtained by Lamson and Edmond ($\times$) the single weighings of Hasselbalch ($\odot$) and some additional embryonic weighings made in the course of later experiments (o).

growth are represented graphically (Fig. 4) it is seen that except for the 17 to 18 day interval there is a rather gradual smooth ascent in the values. Later weighings for 18 day ($\log 18 = 1.255$) old embryos, as shown in Fig. 5, indicate that the average weight at this age is probably higher than was first estimated and that the break in the curve does not represent the usual occurrence. It is suggested that Brody's rhythmic growth curves (3) for the embryonic period were due to similar chance variations, since they do not coincide one with another, and since the figures on which they were based were obtained

as a result of a very small (Lamson and Edmond (4)) or a minimal (Hasselbalch (5)) number of weighings.

Single weighings after incubation conditions had become standardized by the construction of a temperature room as previously described are shown in Fig. 5 for comparison with the standard curve (Fig. 3)., To these are added the figures collected by Brody (3) after Lamson and Edmond, and Hasselbalch.

By the use of the formula the percentage rate of growth may be obtained.

$$\frac{d}{dt}\left[\frac{dw}{W}\right] = \frac{K}{t}$$
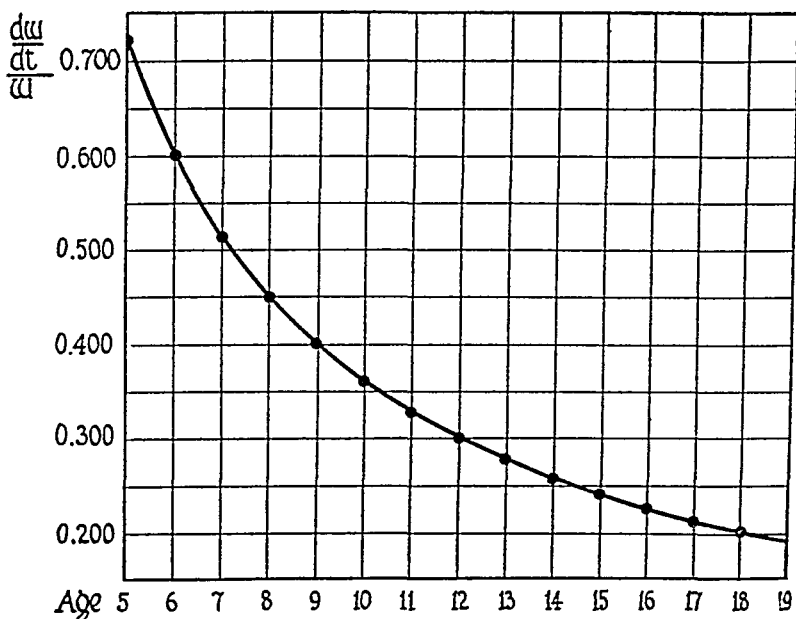
FIG. 6. The percentage growth rate as a function of age (Table I).

where $K = 3.6$. The rate of growth as given by this equation may then be plotted as a function of age (Fig. 6). It is seen that the percentage rate of growth decreases progressively with age. If the rate of growth is used as a criterion of age the velocity of aging (senescence), or in other words, the negative acceleration of the percentage

increase in weight with time (Fig. 7), may be obtained by a further differentiation:

$$\frac{d}{dt}\left[\frac{\dfrac{dw}{W}}{dt}\right] = -\frac{K}{t^2}$$

where $K = 3.6$.

Minot's dicta were that (1) the rate of growth depends on the degree of senescence; (2) senescence is at its maximum in the
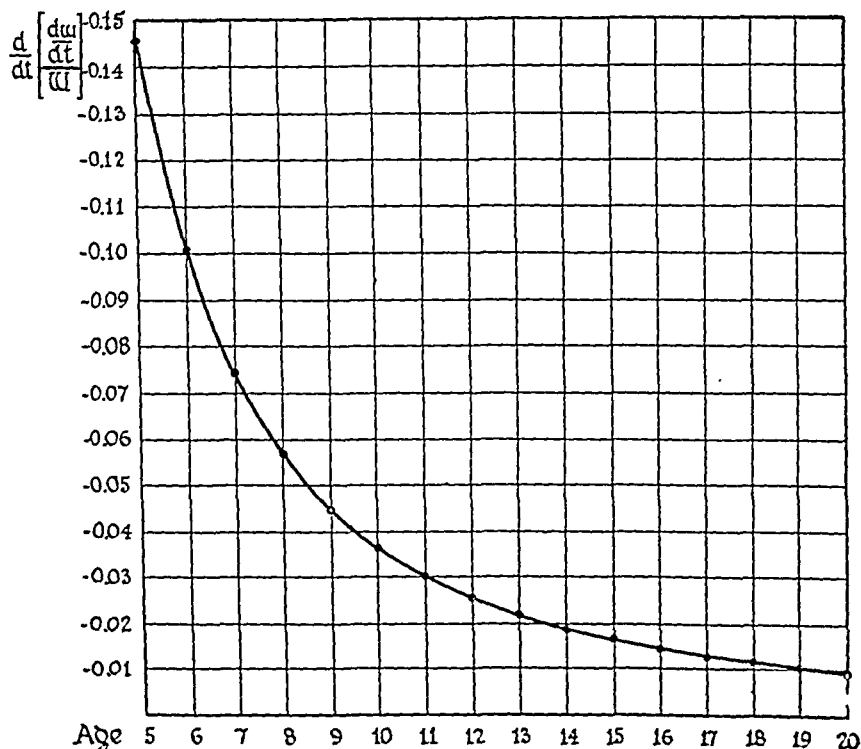
FIG. 7. The negative acceleration of growth as a function of age (Table I).

very young stages; (3) the rate of senescence diminishes with age; and (4) as a corollary from these, natural death is the consequence of cellular differentiation (6). We cannot at present accept this statement as a satisfactory description of senescence, for there is yet no evidence to show that the degree of "aliveness" and velocity of

growth are synonymous. There are other and perhaps more significant phenomena than growth rate which change with age. An organism as a force can only be interpreted in terms of totality of function. Minot's concept that death is the consequence of cellular differentiation seems to be at variance with his other hypothesis. For instance, tissue differentiation, as far as can be judged from its chemical constitution, occurs most rapidly during the latter part of the embryonic period rather than at the beginning. This phase of the subject will be discussed in a later paper.

SUMMARY.

1. The average weights of chicken embryos between 5 and 19 days of incubation as found by over 600 weighings may be expressed by a simple exponential equation,

$$W = K t^{3.6}$$

where $K = 0.668$.

2. The velocity of growth (*i.e.* the percentage increase in mass) is inversely proportional to the incubation age. The product of the two (vt) is a constant (3.6). The negative acceleration of growth likewise decreases with age.

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TEMPERATURE CHARACTERISTIC FOR LOCOMOTOR ACTIVITY IN TENT CATERPILLARS.

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I.

At different temperatures young tent caterpillars (*Malacosoma americanum*) were caused to creep vertically upward upon a thin wooden rod (3 mm. diameter) with evenly roughened surface. The rod was 24 cm. long, and the larva began ascent at the bottom; the mid-region of the rod was graduated, so that the time taken to travel 10 cm. could be accurately observed. Simultaneously, the number of "peristaltic" locomotor waves required to cover the 10 cm. distance was obtained by counting the steps of the anal prolegs. In case the initial or the terminal step failed to coincide precisely with the selected graduation mark, an estimate was made of the fractional step involved. The rod and a thermometer were suspended in a large box which also contained an electric heating device controlled with a rheostat. The box was set up in a room having approximately the desired temperature. For higher temperatures a small dark room was used which could be heated electrically. The animals were adapted to the temperature of the experiment until the rate of creeping became constant.

From the observations taken it was possible to compute: (1) the speed of progression; (2) the frequency of abdominal locomotor waves; (3) the mean amplitude of the step taken by the anal prolegs. We desired to consider these quantities in their relations to temperature, and to compare the critical thermal increment of locomotor activity with that found for some homologous activities.

It was found that variation in speed of creeping could be adequately controlled by the temperature, provided animals of the same size were used. Among such individuals, especially if taken from the

same nest, the rate of creeping is very uniform. We have used caterpillars 1.5 to 2.0 cm. long. About 14 individuals were employed in obtaining 180 measurements. The points plotted in Fig. 3 are each the average of 6 or more very closely concordant determinations.

II.

When the mean speeds of creeping at different constant temperatures are considered in terms of the relationship known to be valid for a number of similar instances,

$$\log_e \frac{\text{velocity at } T_2}{\text{velocity at } T_1} = \frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$
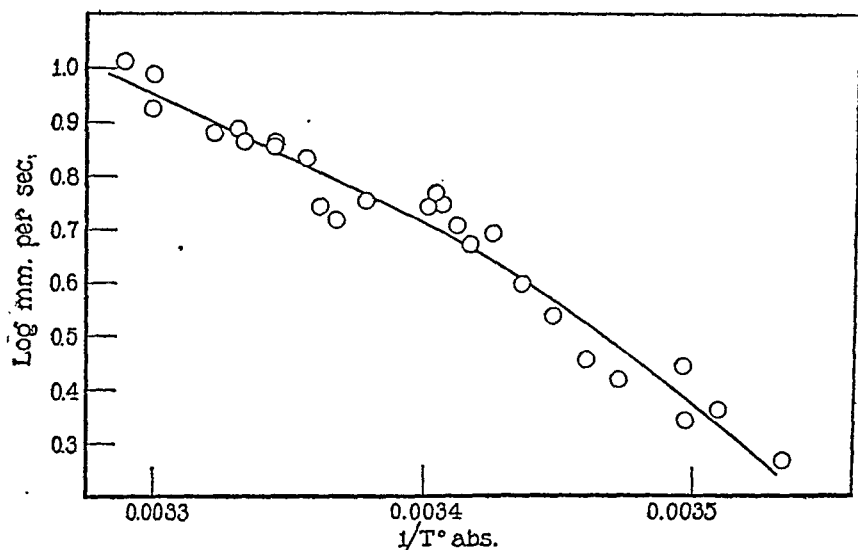
FIG. 1. Mean velocity of creeping is plotted logarithmically against reciprocal of absolute temperature. The relationship is not rectilinear.

the graphs of log velocity *versus* 1/absolute temperature fails to be rectilinear (Fig. 1). No significant value of the critical increment μ can be calculated.

This result finds its explanation in the fact that one of the two components determining the speed of vertical ascension is influenced by the temperature in a special way. The act of creeping is begun by a forward movement of the anal prolegs, initiating a peristaltic

body wave coursing cephalad to the anterior margin of the abdomen; simultaneously the anterior thoracic legs begin their progression movements, the wave of leg activity running posteriorly. The abdominal movements may be timed by counting the steps of the anal prolegs. Between 20° and 30° the number of such steps per 10 cm. distance is sensibly constant. Above 30° there is a tendency for the amplitude of the steps to become less, but the available observations are not numerous because creeping is so often irregular at these higher temperatures. Below 20°, however, the amplitude
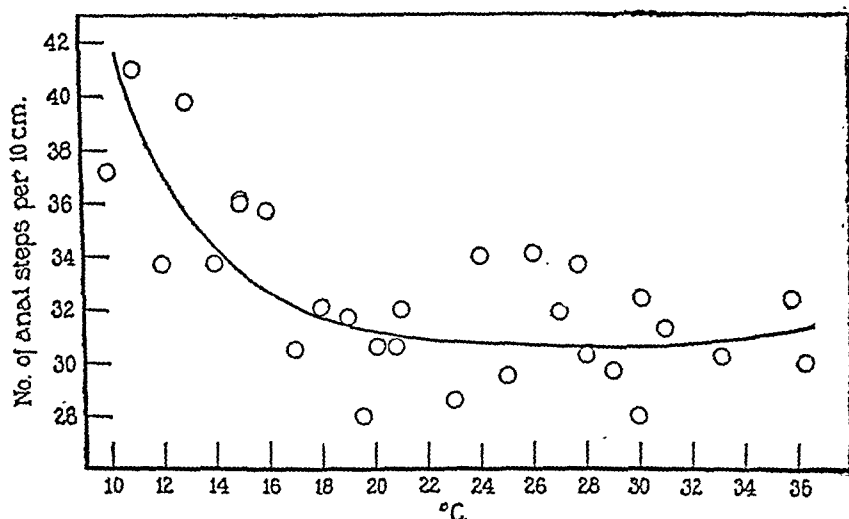
FIG. 2. The number of proleg steps required to ascend 10 cm. is increased as the temperature falls, below 20°.

decreases markedly and regularly as the temperature falls (Fig. 2). It is clear that as a consequence the value of the product (*amplitude of step*) × (*frequency of steps*), which is the velocity of progression, will be abnormally lowered at temperatures less than 20° ± (*cf.* Fig. 1).

The speed of creeping is thus deprived of its possible analytical utility, and it becomes necessary to deal directly with the *frequency* of the locomotor steps. The shortening of the amplitude of the locomotor wave at lower temperatures may have a physical basis in the physiology of the caterpillar's musculature, or it may be condi-

produced in a Pearson schlieren apparatus modified according to Svensson (2). The boundary, after formation in a quartz Tiselius cell, was sharpened by withdrawal through a hollow 4-pronged needle similar to that described by Gutter and Kegeles (3). The diffusion constants for various times were calculated by the method described by Longsworth (4) and extrapolated to infinite time to obtain the true diffusion constant, which was then corrected from 0.9° in buffer to 20.0° in water by the equation

$$D_{20.w} = D_{tb} \times \frac{\eta_{tb}}{\eta_{20.w}} \times \frac{293.2}{273.2 + t} \tag{3}$$

Viscosity—Since the viscosity correction in Equation 3 is quite large, the viscosities of the different buffers were determined in an Ostwald viscometer at the temperature of the diffusion experiments. However, for correcting the sedimentation constant, the temperature dependence of the viscosity for water and buffer was assumed to be the same, since the temperature difference is small.

Concentration—The protein concentration of the solutions was determined from the extinction at 280 mμ in a Beckman DU spectrophotometer, the extinction coefficient of glyceraldehyde-3-phosphate dehydrogenase being taken as 1000 cm.^{2} gm.$^{-1}$ (1).

Apparent Partial Specific Volume—The densities of the protein solution and the buffer were determined pycnometrically and the apparent partial specific volume calculated from the equation

$$\bar{p} = \frac{c - (\rho_s - \rho_b)}{\rho_b c} \tag{4}$$

where c is concentration in gm. per ml.

Electrophoresis—The enzyme solution was subjected to electrophoresis at its isoelectric point, running the current for 3 hours in each direction, and the heterogeneity constant H (5) was calculated from the spreading of the peak by Equation 5.

$$D' = D + \frac{E^2 H^2}{2} t_e \tag{5}$$

where D is the diffusion constant for the protein at the concentration studied, D' is the apparent diffusion constant calculated from the electrophoretic peak, E is the voltage gradient, and t_e is the time of electrophoresis. H is calculated from the slope of D' plotted against t_e (6).

RESULTS AND DISCUSSION

The results of this study are given in Table I and Fig. 1. The protein molecular weight was calculated from the Svedberg equation with the constants given in Table I.

An approximated characterization of the rate of spreading of the sedimenting boundaries was made by calculating an apparent "diffusion coefficient" from the sedimentation diagrams by the height-area method (7), correcting for field (8) and time (9). Although great accuracy cannot be expected from this measurement, the approximate agreement with the value from the diffusion experiments indicates low or negligible polydispersity.

TABLE I

Molecular-Kinetic Constants of D-Glyceraldehyde-3-phosphate Dehydrogenase

$s_{20,w}$.	$7.71 \pm 0.07 \times 10^{-13}$ sec.
$D_{20,w}$ (free diffusion)	$4.97 \pm 0.03 \times 10^{-7}$ cm.^{2} sec.$^{-1}$
$\bar{v}$	0.7253 ± 0.0006 cm.^{3} gm.$^{-1}$
H	0.23×10^{-5} cm.^{2} V^{-1} sec.$^{-1}$
Mol. wt.	$136,900 \pm 1300$ gm. mole^{-1}
$D_{20,w}$ (from sedimentation diagram) ..	$4.24 \pm 0.28 \times 10^{-7}$ cm.^{2} sec.$^{-1}$

For an explanation of the symbols, see the text.
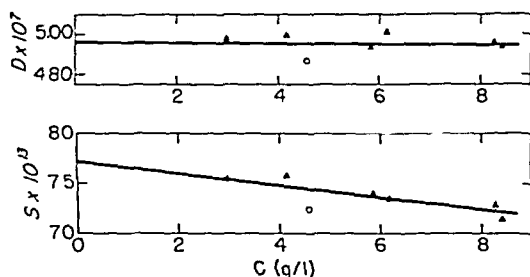
FIG. 1. Diffusion and sedimentation data for glyceraldehyde-3-phosphate dehydrogenase as functions of concentration C (gm. per liter). The lines obtained by the method of least squares may be expressed: $s = 7.71 - 0.061\ C$, and $D = 4.97 - 0.003\ C$. ▲, in phosphate buffer, pH 6.55, ionic strength 0.1; ○, in 0.15 M sodium chloride, pH 6.

There was no separation of components from the electrophoretic peak during the period of the isoelectric point experiment and the relatively small heterogeneity constant calculated under these conditions compares favorably with those reported by Alberty (6) for other purified proteins. The enzyme has also been examined for the presence of other enzymatic activities, specifically those proteins which are known to be isolated under similar conditions. The presence of aldolase activity was tested by Mr. Bernardo Vanderheiden of this Department, by the method of Warburg and Christian (10), the activity found corresponding to 1 part aldolase in 900 parts of triosephosphate dehydrogenase. An assay of α-glycerol

phosphate dehydrogenase was made by the method of Baranowski and Niederland (11) and was found to be in the proportion of 1 part in 2000, the calculation being based on the turnover number given by Baranowski (12).

Measurements of enzymatic activity of the dehydrogenase on its normal substrate were made by the method of Beisenherz et al. (13), which method is essentially the same as that originally reported by Cori et al. (14), except that the enzyme was protected from heavy metal ions by ethylene-diaminetetraacetate ion. The turnover number obtained, 2400 moles per minute per 10^5 gm. of enzyme, agrees with Beisenherz' value. However, since the "second order rate constant" used to obtain the turnover number (calculated according to Cori et al.) is a function of time (14), we have arbitrarily used the constant at zero time. A plot of $1/\log k$ against time was found to be linear, the zero time k depending upon the initial concentration of the substrates for constant enzyme concentration.

In addition to enzymatic contamination of the protein, there is also some contamination by heme compounds. After the eight recrystallizations to which this preparation had been subjected, the extinction at 410 mμ corresponded to only 0.5 per cent hemoglobin by weight.

In view of the lack of evidence for any gross amounts of contamination, and the close agreement of this study with the earlier reported work from this laboratory, the authors have felt justified in using the value of 137,000 as the molecular weight of glyceraldehyde-3-phosphate dehydrogenase in subsequent work.

SUMMARY

The molecular weight of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase has been found to be 137,000 by sedimentation and diffusion. Several criteria of purity, both physical and biochemical, have been applied to the enzyme preparation and have shown that low or negligible amounts of impurities are present.

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SPECIFICITY OF CATHEPSIN C*

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Cathepsin C, one of the proteinases found in animal tissues (1), has been extensively purified from beef spleen (2). Earlier studies showed that at pH 5 crude preparations of this enzyme catalyze the hydrolysis of dipeptide amides at the CO—NH₂ bond (3), and of the ester linkage of dipeptide esters (4). With suitable substrates, the predominant reaction near pH 5 is hydrolysis, whereas near pH 7.5 the enzyme catalyzes replacement reactions leading to the polymerization of the dipeptide units, and yielding long chain peptides (3, 5).

In the present investigation, an attempt was made to define more precisely the effect, on the rate of enzymic action, of changes in the structure of substrates of cathepsin C. It had been demonstrated previously that the enzyme is specific in its action on dipeptide esters (or amides) containing two α-amino acid residues; thus glycyl-L-phenylalanine ethyl ester is readily hydrolyzed at pH 5, whereas β-alanyl-L-phenylalanine ethyl ester is resistant under comparable experimental conditions (4). Also, it had been shown that several amino acid esters were not attacked, and that acylation of the free α-amino group of a sensitive dipeptide amide blocks enzyme action (4). Hence the "backbone" specificity (6) of cathepsin C was considered to be as given in the accompanying formula, where X is either NH₂ or an alkoxy group.

$$
\begin{array}{cc}
\text{R} & \text{R}' \\
| & | \\
\end{array}
$$
$$\text{NH}_2\text{CHCO—NHCHCO——}X$$
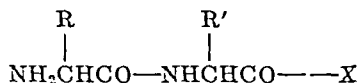
Cathepsin C does not appear to exhibit absolute "side chain" specificity in regard to the nature of the R and R' groups, although the best substrates found previously are those in which the R group is hydrogen or a small aliphatic side chain (methyl, hydroxymethyl), and the R' group is the benzyl or *p*-hydroxybenzyl side chain of L-phenylalanine or of L-tyrosine, respectively. (The corresponding derivatives of D-phenylalanine are resistant (5).) Thus, glycyl-L-phenylalaninamide or glycyl-L-tyrosina-

mide or the corresponding ethyl esters are suitable substrates. It should be added, however, that glycylglycine ethyl ester and glycyl-L-leucine ethyl ester[1] are also readily hydrolyzed by a highly purified preparation of cathepsin C.

To investigate further the structural requirements in substrates of cathepsin C, a series of dipeptide amides and esters was prepared in which the R' group was the side chain of L-tyrosine or L-phenylalanine, and other structural features of the compounds were varied.

Attention may be given first to the action of cathepsin C at pH 5, since here it is possible to study the rate of hydrolysis under conditions in which the extent of transamidation is negligible and the substrates are stable in the absence of added enzyme. The data are summarized in Table I. It will be noted that, in general, the comparable amides and esters are hydrolyzed at similar rates, although in some instances the esters are cleaved more rapidly. Thus, glycyl-L-tyrosinamide and glycyl-L-tyrosine ethyl ester are hydrolyzed at approximately the same rate at pH 5, whereas sarcosyl-L-tyrosinamide is split more slowly than is sarcosyl-L-tyrosine ethyl ester. It was found previously (4) that crude preparations of cathepsin C act more rapidly on glycylglycine ethyl ester than on glycylglycinamide.

A further point of general interest is that the action of cathepsin C is similar on pairs of substrates that differ only in having an L-phenylalanine or an L-tyrosine residue at the sensitive linkage. As may be seen from the data in Table I, methylation of the phenolic hydroxyl group of the L-tyrosine residue also does not alter the rate of enzymic action.

The replacement of the glycyl residue of glycyl-L-tyrosinamide, glycyl-L-phenylalaninamide, glycyl-L-tyrosine ethyl ester, or glycyl-L-phenylalanine ethyl ester by a sarcosyl residue in all cases leads to a marked diminution of the rate of enzymic hydrolysis at pH 5. It may be concluded, therefore, that the enzymic action is inhibited by the introduction of a methyl group at the terminal α-amino group of substrates such as glycyl-L-tyrosinamide. However, the slower hydrolysis of the sarcosyl compounds cannot be interpreted to indicate that a primary α-amino group is essential for the action of cathepsin C, since the replacement of the glycyl residue of glycyl-L-tyrosinamide or glycyl-L-tyrosine ethyl ester by an L-prolyl residue gives substrates that are hydrolyzed at essentially the same rate as the glycyl compounds, although the prolylphenylalanine derivatives are split somewhat more slowly than is glycyl-L-phenylalaninamide or glycyl-L-phenylalanine ethyl ester. Since the N-dimethylglycyl compounds were not available, it cannot be stated at present whether the enzyme action is limited to substrates that have an N-terminal amino or

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[1] M. J. Mycek and J. S. Fruton, unpublished observations.

imino acid. As noted above, acylation of the terminal α-amino group blocks the action of the enzyme.   In this connection, it may be added that acetyl-L-phenylalanine ethyl ester is resistant to the hydrolytic action of cathepsin C at pH 5 (Table I).

TABLE I

Action of Cathepsin C on Peptide Amides and Peptide Esters near pH 5

The first column denotes the N-terminal amino acid and other substituents on the compounds indicated at the top of the next four columns.   The experimental conditions were as given in the experimental section.

Substituent	Hydrolysis in 2 hrs			
	L Tyrosinamide	L Phenylalaninamide	L-Tyrosine ethyl ester	L-Phenylalanine ethyl ester
	μmoles per ml	μmoles per ml	μmoles per ml.	μmoles per ml.
Glycyl-	19	21	18	22
Glycyl-p methoxy-		21		
Sarcosyl- ..	3	2	10	8
L-Prolyl- . .	16	15*	19	14*
Glycyl-N-methyl- .		0†		
L-Alanyl- .	12		14	
D-Alanyl-			6	
α-Aminoisobutyryl-			6	7
L-Seryl-	12			
L-Leucyl-				5
D-Leucyl-				1‡
L-Lysyl-	0	0	0	0
ϵ-Acetyl-L-lysyl-	2	2	2	2
ϵ-Benzoyl-L-lysyl-	0	0	0	0
Glycylglycyl-				0
Acetyl- .				0§

* L-Prolyl-L-phenylalanine crystallized.

† This value was determined by means of the Grassmann-Heyde titration method.

‡ Tested with 1 4 units of cathepsin C, and the measured rate divided by 4 to facilitate comparison

§ Tested in the presence of 30 per cent methanol; under these conditions, glycyl-L-phenylalanine ethyl ester was hydrolyzed at a rate of 6 μmoles per hour.

It was of interest to find that glycyl-N-methyl-L-phenylalaninamide is resistant to the action of cathepsin C, demonstrating that the introduction of a methyl group at the peptide bond between the 2 α-amino acid residues blocks the hydrolysis of the CO—NH$_2$ linkage.   Thus, although the enzyme does not catalyze the hydrolysis of the bond joining the 2 amino acid residues, substitution of the N atom of this bond decisively interferes with the action of cathepsin C.

In studying the effect of changes in the "side chain" of the N-terminal amino acid on the rate of enzymic action, the glycyl residues of the standard substrates were replaced by the following: L-alanyl, D-alanyl, α-aminoisobutyryl, L-seryl, L-leucyl, D-leucyl, L-lysyl, ε-acetyl-L-lysyl, ε-benzoyl-L-lysyl. All the resulting compounds were hydrolyzed more slowly than the corresponding glycyl compounds or were resistant to hydrolysis (Table I). Although replacement of the glycyl residue of glycyl-L-tyrosinamide or glycyl-L-phenylalaninamide by an L-alanyl or L-seryl residue diminishes only slightly the rate of enzyme action, the introduction of the side chain of a larger L-amino acid residue, as in L-leucyl-L-phenylalanine ethyl ester, causes a marked decrease in the rate. With a bulkier neutral side chain such as ε-acetylamino-n-butyl or ε-benzoylamino-n-butyl on the N-terminal residue, the rate of hydrolysis at pH 5 is decreased even further. It is of interest that, although the ε-acetyllysyl derivatives are hydrolyzed slowly under the conditions of these experiments, the corresponding lysyl compounds are resistant to enzyme action. Apparently, the presence of the free ε-amino group in the substrate makes the amide or ester bond less sensitive to attack by cathepsin C. It should be added, however, that, under the usual experimental conditions, substances such as L-lysyl-L-tyrosinamide or L-lysyl-L-phenylalaninamide are not inhibitors of cathepsin C when glycyl-L-tyrosinamide or glycyl-L-phenylalaninamide are substrates (Table II). Also, L-lysyl-L-tyrosine ethyl ester is not hydrolyzed to an appreciable extent by cathepsin C at the linkage between the lysyl and tyrosyl residues, to form free lysine; this was demonstrated by paper chromatography with n-butanol-acetic acid-water (8:2:1) as the solvent.

Experiments were also performed with many of the above derivatives of glycyl-L-tyrosinamide, glycyl-L-phenylalaninamide, glycyl-L-tyrosine ethyl ester, and glycyl-L-phenylalanine ethyl ester to determine their susceptibility to the action of cathepsin C at pH values near 7.5. At this pH, at least three possible reactions may occur: enzyme-catalyzed hydrolysis of the sensitive linkage, enzyme-catalyzed replacement reactions, and non-enzymic cyclization to form diketopiperazines. As judged by the rate of ammonia liberation from dipeptide amides or the disappearance of dipeptide esters, the amides are more stable in the absence of added enzyme at pH 7.5 than are the corresponding esters (Table III). It will be noted from Table III that, if allowance is made for the non-enzymic reaction of the compounds tested at pH 7.5, the relative effects of cathepsin C at this pH are roughly comparable to those observed at pH 5. This is concordant with the view (3) that the catalysis of transamidation at pH 7.5 is effected by the same enzyme that catalyzes hydrolysis at pH 5. Among the compounds listed in Table III, and not previously tested with cathepsin C at pH 7.5, the only ones which appeared to give an insoluble polymer

analogous to that obtained from glycyl-L-phenylalaninamide or glycyl-L-tyrosinamide (3, 5) were glycyl-O-methyl-L-tyrosinamide and ε-acetyl-L-lysyl-L-phenylalaninamide; with the last named compound, the transamidation reaction was slow and the extent of precipitation of presumed polymer was very slight.

It will be noted from Table II that, when glycyl-L-phenylalaninamide or glycyl-L-tyrosinamide was subjected to the action of cathepsin C at pH 7.6

TABLE II

Effect of Lysyl Peptides on Action of Cathepsin C

All reaction flasks contained either 0.05 M glycyl-L-tyrosinamide or 0 05 M glycyl-L-phenylalaninamide. L-Lysyl-L-phenylalaninamide or L-lysyl-L-tyrosinamide was added at a concentration of 0 05 M. In the experiments with glycyl-L-tyrosinamide, 0.2 cathepsin unit was present per 2 ml.; with glycyl-L-phenylalaninamide, 0.16 unit was used. The other experimental conditions were as given in the experimental section.

Substances present	Ammonia liberation per ml.			
	pH 5.1	pH 7.6		
	2 hrs.	1 hr.	2 hrs.	4 hrs.
	μmoles	μmoles	μmoles	μmoles
Glycyl-L-tyrosinamide	12	14*	18*	22*
" + L-lysyl-L-phenylalaninamide	12	19	27	36*
Glycyl-L-tyrosinamide + L-lysyl-L-tyrosinamide	12	19	27	32*
Glycyl-L-phenylalaninamide	10	9*	12*	16*
" + L-lysyl-L-phenylalaninamide	11	12	17*	24*
Glycyl-L-phenylalaninamide + L-lysyl-L-tyrosinamide	9	13	19*	27*

* A gelatinous precipitate was present.

in the presence of L-lysyl-L-phenylalaninamide or L-lysyl-L-tyrosinamide, the rate of ammonia liberation was considerably greater than that observed in the control experiments. Also, the appearance of the polymeric peptides formed from glycyl-L-phenylalaninamide and glycyl-L-tyrosinamide was delayed in the presence of the lysyl compounds. These observations are analogous to those made previously (3) upon the addition of substances such as L-argininamide, and indicate that, although the lysyl compounds are not substrates of cathepsin C, they can serve as replacement agents in transamidation reactions catalyzed by the enzyme. Since the pK' values of the α- and ε-amino groups of these compounds are approximately 8 and

TABLE III

Action of Cathepsin C on Peptide Amides and Peptide Esters near pH 7.5

The data are given in micromoles of ammonia liberated or ester disappeared per 2 ml. of reaction mixture. The numbers in parentheses denote the extent of reaction in the absence of added enzyme. Where no figures are given for control experiments, the compound was stable in the absence of enzyme. Except where otherwise indicated, 0.35 cathepsin unit was used per 2 ml. of reaction mixture. The other experimental conditions were as given in the experimental section.

Compound tested	Extent of reaction			
	0.5 hr.	1 hr.	2 hrs.	4 hrs.
	μmoles	μmoles	μmoles	μmoles
Glycyl-L-tyrosinamide		32*		46*
Glycyl-O-methyl-L-tyrosinamide		33*		52*
Glycyl-L-tyrosine ethyl ester	51 (26)	63* (38)	68* (48)	
Sarcosyl-L-tyrosinamide			8 (3)	15 (5)
Sarcosyl-L-phenylalaninamide			8 (3)	15 (6)
Sarcosyl-L-tyrosine ethyl ester	22 (17)	35 (26)	51 (40)	
Sarcosyl-L-phenylalanine ethyl ester	19 (13)	32 (20)	41 (29)	
L-Prolyl-L-tyrosinamide		31†	62†	80†
L-Prolyl-L-phenylalaninamide		21‡	49‡	75‡
L-Prolyl-L-tyrosine ethyl ester	19 (5)	29 (9)	69 (13)	
L-Prolyl-L-phenylalanine ethyl ester	10 (4)	23‡ (7)	48‡ (9)	
α-Aminoisobutyryl-L-tyrosine ethyl ester		12 (7)	24 (14)§	
α-Aminoisobutyryl-L-phenylalanine ethyl ester		16 (11)	28 (19)§	
L-Lysyl-L-tyrosinamide			0 (2)	2 (2)
L-Lysyl-L-phenylalaninamide			2 (2)	3 (3)
" (1.4 cathepsin units)			2 (2)	3 (3)
L-Lysyl-L-tyrosine ethyl ester	8 (9)	28 (27)	39 (40)	
" (1.4 cathepsin units)	8 (9)	26 (27)	37 (40)	
L-Lysyl-L-phenylalanine ethyl ester	15 (13)	32 (31)	44 (43)	
ε-Acetyl-L-lysyl-L-phenylalaninamide			2	4
" (1.4 cathepsin units)			7	13*
ε-Acetyl-L-lysyl-L-tyrosine ethyl ester	10 (6)	25 (18)	36 (32)	
ε-Acetyl-L-lysyl-L-phenylalanine ethyl ester	10 (8)		32 (28)	
Glycylglycyl-L-phenylalanine ethyl ester		3	6	

* A gelatinous precipitate was present.

† This reaction was followed by the Conway and Grassmann-Heyde methods, and the ammonia liberation was equivalent to the increase in titratable acidity.

‡ L-Prolyl-L-phenylalanine crystallized from the reaction mixture.

§ Diketopiperazine crystallized from the reaction mixture.

10.5, respectively, it may be expected that such transamidation reactions largely involve the participation of the α-amino group of the lysyl residue.

Of special interest was the finding that the dipeptide derivatives con-

taining an *N*-terminal proline, although rapidly attacked by cathepsin C at pH 7.5, did not undergo any measurable transamidation. In the case of the derivatives of L-prolyl-L-phenylalanine, a crystalline product appeared during the enzyme reaction, and was identified as the sparingly soluble free dipeptide (7). When the action of cathepsin C on L-prolyl-L-tyrosinamide was followed at pH 7.5 both by measurement of ammonia liberation and by titration with alcoholic KOH, the ammonia liberation was stoichiometrically equivalent to the increase in titratable acidity. As shown previously (3), when transamidation occurs with substrates such as glycyl-L-phenylalaninamide, the extent of ammonia liberation is greater than the increase in titratable acidity. It may be concluded, therefore, that the action of cathepsin C on the prolyl dipeptide derivatives tested is restricted to hydrolysis, and that these prolyl compounds do not serve as replacement agents to a measurable extent. In this connection, it may be added that other studies in this laboratory have shown that, in the action of papain on carbobenzoxyglycinamide at pH 7.5, dipeptides such as L-leucylglycine are very effective replacement agents, whereas L-prolylglycine is completely ineffective in this regard.[1] The failure of prolyl compounds to serve as replacement agents may be due, in part, to the fact that the pK' of the immonium group in such compounds is near 9 (8), although steric factors may also be involved.

The data in Table III also are of interest in relation to the relative stability of dipeptide derivatives at pH 7.5, in the absence of added enzyme. The fact that the prolyl derivatives are more stable than the comparable glycyl or sarcosyl compounds is noteworthy in view of the known tendency of compounds such as glycyl-L-proline, L-prolyl-L-proline, and glycyl-L-prolinamide to undergo cyclization with great ease (9). It would appear that the formation of diketopiperazines is favored by the presence of a proline residue in an amino acylprolyl compound rather than at the *N*-terminal position.

A number of compounds were not tested at pH 7.5, because they were sparingly soluble in water at this pH. They were ε-acetyl-L-lysyl-L-tyrosinamide, all the ε-benzoyl derivatives of the lysine compounds, L-leucyl-L-phenylalanine ethyl ester, and D-leucyl-L-phenylalanine ethyl ester.

DISCUSSION

The studies described in the present communication, together with earlier work on cathepsin C, justify the conclusion that this enzyme is adapted to the hydrolysis of amide or ester bonds of dipeptide amides or dipeptide esters containing two α-amino acid residues. Sufficient data are not available about the action of cathepsin C on CO—NH bonds joining two α-amino acids to permit the conclusion that this enzyme can cleave dipep- .

tide units from the amino end of a long peptide chain; studies on the action of cathepsin C on proteins and long chain synthetic peptides are in progress. If the specificity observed with synthetic substrates applies to the action of the enzyme on proteins, cathepsin C may prove to be a useful addition to the proteolytic enzymes used as reagents for the selective cleavage of proteins. From the studies reported in this paper, it may be expected that cathepsin C would be more effective in attacking peptide chains in which the N-terminal amino acid is proline or one that bears a small aliphatic side chain (as in glycine, alanine, serine).

One may venture to interpret the effect, on the rate of enzyme action, of structural changes in substrates of cathepsin C in terms of a hypothesis concerning the stereochemistry of the enzyme-substrate interaction. It seems likely that the enzyme requires, in its substrates, a free terminal α-amino or α-imino group, and it may be assumed that the combination of cathepsin C with a substrate involves such a group, as well as the carbonyl group that participates in the sensitive linkage. The inability of cathepsin C to hydrolyze β-alanyl-L-phenylalanine ethyl ester (4) indicates that the distance between the terminal amino group and the sensitive carbonyl group is critical for enzymic action. It may be surmised that, in the combination of the enzyme with the substrate in a manner leading to catalysis, the portion of the substrate molecule between (and including) these groups is held in a specific conformation. One possibility is that, in the enzyme-substrate complex, this portion of the substrate molecule is in a form tending toward a hexagon, as shown in Fig. 1, a.

If it is assumed that the "active center" of the enzyme interacts with the substrate by approaching it from above the plane of the hexagon (as drawn in Fig. 1, a), then it seems possible to interpret several aspects of the specificity of cathepsin C. It may be expected that the presence of D-amino acid residues at the N-terminal position (Fig. 1, b), or contributing the R′ group (Fig. 1, c), would tend to prevent a close fit of enzyme and substrate. Thus D-leucyl-L-phenylalanine ethyl ester is hydrolyzed very slowly (Table I), and glycyl-D-phenylalaninamide is resistant to enzyme action (5). If the side chain group is small, as in D-alanine, hydrolysis of D-alanyl-L-tyrosine ethyl ester is observed, but this is slower than the hydrolysis of L-alanyl-L-tyrosine ethyl ester (Table I). It is perhaps significant that D-alanyl-L-tyrosine ethyl ester is hydrolyzed at approximately the same rate as is α-aminoisobutyryl-L-tyrosine ethyl ester (Table I), which has methyl groups projecting above and below the plane of the hexagon (Fig. 1, d).

The effect of large R groups of N-terminal L-amino acid residues in decreasing the sensitivity of substrates of cathepsin C may perhaps be attributed to steric hindrance of the enzyme-substrate interaction as a con-

sequence of the free rotation about the bonds of the side chain (*e.g.*, of the ε-acetyl-L-lysyl residue). However, where such free rotation is not possible, as in L-prolyl, the approach of the enzyme may not be hindered, thus explaining the ready hydrolysis of the prolyl compounds. It may be surmised that the slower hydrolysis of the sarcosyl compounds, as compared to the analogous L-prolyl compounds, is a consequence of steric hindrance caused by an *N*-methyl group that can assume a position above the plane of the postulated hexagon. The inability of cathepsin C to act on glycyl-*N*-methyl-L-phenylalaninamide may be due to steric hindrance caused by substitution at the CO—NH bond between the 2 amino acid residues. This substitution may interfere with the folding of the peptide chain into a conformation required for successful interaction with the enzyme, and may
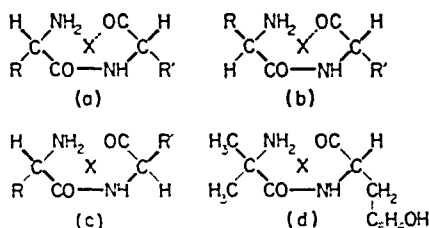
Fig. 1. Postulated folding of dipeptide derivatives in their interaction with cathepsin C. The heavy lines denote bonds in front of the plane of the paper, whereas the light lines denote bonds behind the plane of the paper. The dash lines represent the bond cleaved by the enzyme. *a*, a dipeptide derivative composed of two L-amino acid residues; *b*, a dipeptide derivative in which the *N*-terminal amino acid has a D configuration; *c*, a dipeptide derivative in which the R′ group belongs to a D-amino acid; *d*, a derivative of α-aminoisobutyryl-L-tyrosine.

prevent an interaction, by hydrogen bond formation, with the CO—NH group of the substrate.

Experiments now in progress are designed to determine the magnitudes of $(k_2 + k_3)/k_1$ and k_3 in the reactions

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_3}{\longrightarrow} E + \text{products}$$
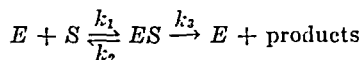
where E is cathepsin C and the substrate (S) is one of the series of dipeptide derivatives found to be hydrolyzed by the enzyme. It may be hoped that such data on the effect of structural changes in the substrate on the relative magnitude of K_m and k_3 will throw further light on the mechanism of cathepsin C action.

EXPERIMENTAL

In the conduct of the enzyme experiments, the pH was maintained at pH 5.0 to 5.2 with 0.1 M citrate buffer and at pH 7.4 to 7.6 with 0.1 M

phosphate buffer. Cysteine (0.004 M) was used as activator. The cathepsin C preparation was kindly provided by Dr. de la Haba, and had a specific activity [C. U.]$_{mg.N}^{GPA}$ = 175, in the terms defined previously (1). In most of the experiments reported in this communication, the enzyme concentration was 0.35 cathepsin unit per 2 ml. of reaction mixture; in some instances, as indicated in Table I, the enzyme concentration was increased to 1.4 units per 2 ml. The substrate concentration was 0.05 M in all cases. The temperature was maintained at 37.0°. The rate of enzyme action on the peptide amides was followed by measurement of the extent of ammonia liberation in Conway microdiffusion vessels (10). The rate of disappearance of the peptide esters was followed by a modification of the hydroxamic acid method of Hestrin (4, 11). In several experiments, the Grassmann-Heyde titration method (12) also was employed. In all cases, control experiments were performed to determine the lability of the substrate, in the absence of added enzyme, at the pH and temperature of the enzyme test. Where no data are given for such control experiments, the substrates were found to be stable during the period of the enzyme experiment.

To facilitate comparison of the rates of hydrolysis of the various compounds tested near pH 5, the data in Table I denote the micromoles of substrate cleaved per ml. during the first 2 hours of the incubation, although samples were withdrawn for analysis at other time intervals (1 hour, 4 hours) as well. It was found in most cases that, during the initial 2 hour interval, and at the initial substrate concentration of 0.05 M, the rate at pH 5 was linear with time within the precision of the analytical methods employed. It was assumed that the extent of transamidation at pH 5 was sufficiently small so that it could be neglected (3).

Glycyl-L-tyrosinamide acetate, glycyl-L-phenylalaninamide acetate, L-alanyl-L-tyrosinamide acetate, and glycylglycyl-L-phenylalanine ethyl ester acetate were prepared as described previously (5). The synthesis of glycyl-L-phenylalanine ethyl ester hydrochloride and L-seryl-L-tyrosinamide hydrochloride also has been described (4).

For the synthesis of the other compounds tested as substrates, the general method of Vaughan and Osato (13) was used. The coupling reaction was usually performed by first preparing at −5° the requisite mixed anhydride from the carbobenzoxy amino acid (0.01 mole) and isobutylchlorocarbonate (0.01 mole) in the presence of triethylamine (0.01 mole) and toluene (20 ml.). After 15 minutes, a chilled mixture of the amino acid ethyl ester hydrochloride (0.01 mole), triethylamine (0.01 mole), and chloroform (20 ml.) was added. The reaction mixture was left at room temperature overnight, washed with water, dilute bicarbonate solution, and water; the organic layer was dried with exsiccated Na_2SO_4, and concentrated in vacuo to yield the desired carbobenzoxy dipeptide ester. For conversion of the

ester to the corresponding amide, the compound (about 0.01 mole) was dissolved in 25 ml. of methanol previously saturated with dry NH_3 at 0°, and the solution was kept at room temperature for 2 days. The solution was then concentrated *in vacuo* to give the carbobenzoxy dipeptide amide. The removal of the carbobenzoxy group was performed in the usual manner by catalytic hydrogenolysis in the presence of palladium-black and the equivalent amount of 10 N hydrochloric or of glacial acetic acid; ethanol was used as the solvent.

Glycyl-L-tyrosine Ethyl Ester Hydrochloride—This compound was prepared by hydrogenolysis of 1 gm. of the carbobenzoxy derivative (14). Yield, 0.6 gm.; $[\alpha]_D^{22}$ +17.1° (2 per cent in water).

$C_{13}H_{19}O_4N_2Cl$ (302.8). Calculated, N 9.3; found, N 9.5

The acetate of this dipeptide ester has been described previously (4).

Glycyl-O-methyl-L-tyrosinamide Acetate—3 gm. of O-methyl-L-tyrosine (15) were converted to the ethyl ester hydrochloride in the usual manner. Yield, 92 per cent; m.p., 202°; $[\alpha]_D^{22}$ −4.9° (2 per cent in water).

$C_{12}H_{18}O_2NCl$ (259.7). Calculated, N 5.4; found, N 5.4

The coupling reaction gave an oily carbobenzoxy dipeptide ester, which was converted to the crystalline amide with an over-all yield of 61 per cent; m.p., 132°.

$C_{20}H_{23}O_5N_3$ (385.4). Calculated, N 10.9; found, N 10.8

Hydrogenolysis gave the dipeptide amide acetate; yield, 63 per cent; $[\alpha]_D^{22}$ +39.5° (2 per cent in water).

$C_{14}H_{21}O_5N_3$ (311.3). Calculated, N 13.5; found, N 13.4

Sarcosyl-L-tyrosinamide Hydrochloride—Sarcosine (0.1 mole) was carbobenzoxylated in the usual manner to give the oily carbobenzoxysarcosine in a yield of 97 per cent. The coupling reaction gave the carbobenzoxy dipeptide ester in a yield of 64 per cent; m.p., 136°.

$C_{22}H_{26}O_6N_2$ (414.5). Calculated, N 6.8; found, N 6.7

The amide was obtained in a yield of 85 per cent; m.p., 122–124°.

$C_{20}H_{23}O_5N_3$ (385.4). Calculated, N 10.9; found, N 11.0

Hydrogenolysis of 1.3 gm. of the amide gave the desired product in a yield of 87 per cent; $[\alpha]_D^{22}$ +31.5° (2 per cent in water).

$C_{12}H_{18}O_2N_3Cl$ (287.8). Calculated, N 14.6; found, N 14.3

Sarcosyl-L-tyrosine Ethyl Ester Hydrochloride—Hydrogenolysis of 2 gm. of the carbobenzoxy compound gave the product in 96 per cent yield. The

substance was recrystallized from methanol-ether; $[\alpha]_D^{22}$ +11.0° (2 per cent in water).

$C_{14}H_{21}O_4N_2Cl$ (316.8). Calculated, N 8.8; found, N 8.8

Sarcosyl-L-phenylalaninamide Hydrochloride—The carbobenzoxy dipeptide ester was obtained as an oil, and was converted to the amide (over-all yield, 49 per cent); m.p., 134°.

$C_{20}H_{23}O_4N_3$ (369.4). Calculated, N 11.4; found, N 11.2

Hydrogenolysis gave the dipeptide amide hydrochloride in 84 per cent yield; $[\alpha]_D^{22}$ +25.4° (2 per cent in water).

$C_{12}H_{18}O_2N_3Cl$ (271.8). Calculated, N 15.5; found, N 15.2

Sarcosyl-L-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of the oily carbobenzoxy compound gave the product in 82 per cent yield; $[\alpha]_D^{22}$ +4.7° (2 per cent in water).

$C_{14}H_{21}O_3N_2Cl$ (300.8). Calculated, N 9.3; found, N 9.3

L-*Prolyl-L-tyrosinamide Hydrochloride*—The oily carbobenzoxy dipeptide ester was converted to the amide (over-all yield, 52 per cent); m.p., 172–173°.

$C_{22}H_{25}O_5N_3$ (411.5). Calculated, N 10.2; found, N 10.0

Hydrogenolysis gave the dipeptide amide in non-crystalline form (yield, 99 per cent). Despite repeated precipitation from methanol-ether, the substance could not be obtained in a completely pure state.

$C_{14}H_{20}O_3N_3Cl$ (313.8). Calculated, N 13.4; found, N 12.7

L-*Prolyl-L-tyrosine Ethyl Ester Hydrochloride*—Hydrogenolysis of the oily carbobenzoxy compound gave a non-crystalline solid in 80 per cent yield; $[\alpha]_D^{22}$ −29.3° (2 per cent in water).

$C_{16}H_{23}O_4N_2Cl$ (342.8). Calculated, N 8.2; found, N 7.9

L-*Prolyl-L-phenylalaninamide Hydrochloride*—The oily carbobenzoxy dipeptide ester was converted to the amide (over-all yield, 68 per cent); m.p., 180–181°.

$C_{22}H_{25}O_4N_3$ (395.5). Calculated, N 10.6; found, N 10.4

Hydrogenolysis gave a non-crystalline product; yield, 94 per cent; $[\alpha]_D^{22}$ −15.8° (2 per cent in water).

$C_{14}H_{20}O_2N_3Cl$ (297.7). Calculated, N 14.1; found, N 13.8

L-*Prolyl-L-phenylalanine Ethyl Ester Hydrochloride*—Hydrogenolysis of

the oily carbobenzoxy compound gave the desired product in crystalline form; yield, 75 per cent; $[\alpha]_D^{22}$ $-40.9°$ (2 per cent in water).

$$C_{16}H_{23}O_3N_2Cl \text{ (326.8). Calculated, N 8.6; found, N 8.5}$$

When 32.7 mg. of this substance were subjected to enzymic hydrolysis by cathepsin C at pH 5 under the usual conditions, a crystalline precipitate appeared within 90 minutes. After 4 hours, 55 per cent of the ester had disappeared, as judged by the hydroxamic acid method, and the crystals were filtered off, washed with water, and dried. Yield, 12.5 mg.; m.p., 242–244°, with decomposition. This product is L-prolyl-L-phenylalanine (7).

$$C_{14}H_{18}O_3N_2 \text{ (262.3). Calculated, N 10.7; found, N 10.8}$$

Glycyl-N-methyl-L-phenylalaninamide Hydrochloride—N-Methyl-L-phenylalanine was prepared from 3.3 gm. of D-phenylalanine in a manner similar to that described for the conversion of L-methione to N-methyl-D-methionine (16). Yield, 1.9 gm. (53 per cent); $[\alpha]_D^{22}$ $+48.4°$ (2 per cent in 0.13 N NaOH). Fischer (17) has reported $[\alpha]_D^{15}$ $+49.7°$ (2 per cent in 0.1 N NaOH) for this compound. It was converted in the usual manner to the ethyl ester hydrochloride. Yield, 96 per cent; m.p., 133°; $[\alpha]_D^{22}$ $+ 7.8°$ (2 per cent in water).

$$C_{12}H_{18}O_2NCl \text{ (243.7). Calculated, N 5.7; found, N 5.3}$$

The oily carbobenzoxy dipeptide ester was converted to the amide which crystallized with difficulty. Over-all yield, 43 per cent; m.p., 130°.

$$C_{20}H_{23}O_4N_3 \text{ (369.4). Calculated, N 11.4; found, N 11.7}$$

Hydrogenolysis gave the dipeptide amide hydrochloride, which was recrystallized from a mixture of methanol, acetone, and ethyl acetate. Yield, 61 per cent; $[\alpha]_D^{22}$ $-19.6°$ (2 per cent in water).

$$C_{12}H_{18}O_2N_3Cl \text{ (271.8). Calculated, N 15.5; found, N 15.5}$$

This compound was found to decompose with the liberation of ammonia when in contact with the carbonate used in the Conway method.

Glycyl-N-methyl-L-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of the oily carbobenzoxy compound gave the product in poor yield (25 per cent).

$$C_{14}H_{21}O_3N_2Cl \text{ (300.8). Calculated, N 9.3; found, N 9.3}$$

L-*Alanyl-L-tyrosine Ethyl Ester Hydrochloride*—Hydrogenolysis of the carbobenzoxy compound (18) gave the desired product in 85 per cent yield; $[\alpha]_D^{25}$ $+6.4°$ (2 per cent in water).

$$C_{14}H_{21}O_4N_2Cl \text{ (316.8). Calculated, N 8.8; found, N 8.6}$$

D-*Alanyl-*L-*tyrosine Ethyl Ester Hydrochloride*—The coupling product was obtained in 78 per cent yield; m.p., 105–107°.

$C_{22}H_{26}O_6N_2$ (414.5). Calculated, N 6.8; found, N 6.7

Hydrogenolysis gave the dipeptide ester hydrochloride in 90 per cent yield; $[\alpha]_D^{25}$ +4.9° (2 per cent in water).

$C_{14}H_{21}O_4N_2Cl$ (316.8). Calculated, N 8.8; found, N 8.5

α-*Aminoisobutyryl-*L-*tyrosine Ethyl Ester Hydrochloride*—Carbobenzoxy-α-aminoisobutyric acid was prepared in the usual manner; yield, 53 per cent; m.p., 64–66°.

$C_{12}H_{15}O_4N$ (237.3). Calculated, N 5.9; found, N 5.8

The oily carbobenzoxy dipeptide ester was subjected to hydrogenolysis, yielding a non-crystalline solid in 45 per cent yield; $[\alpha]_D^{22}$ −9.1° (2 per cent in water).

$C_{15}H_{23}O_4N_2Cl$ (330.8). Calculated, N 8.5; found, N 8.4

α-*Aminoisobutyryl-*L-*phenylalanine Ethyl Ester Hydrochloride*—The coupling product was obtained in 35 per cent yield; m.p., 94–95°.

$C_{23}H_{28}O_5N_2$ (412.5). Calculated, N 6.8; found, N 6.8

Hydrogenolysis gave the dipeptide ester hydrochloride in non-crystalline form. Yield, 77 per cent; $[\alpha]_D^{22}$ −18.1° (2 per cent in water).

$C_{15}H_{23}O_3N_2Cl$ (314.8). Calculated, N 8.9; found, N 9.0

On incubation of 31.5 mg. of this compound dissolved in 2 ml. at pH 7.5 and 37° for 4 hours, 7.6 mg. of a crystalline precipitate were obtained (m.p., 290–292°, decomposition); its nitrogen analysis agrees with that of α-amino-isobutyryl-L-phenylalanine diketopiperazine.

$C_{13}H_{16}O_3N_2$ (232.3). Calculated, N 12.1; found, N 12.2

L-*Leucyl-*L-*phenylalaninamide Acetate*—The coupling reaction gave an oily carbobenzoxy dipeptide ester, which was converted to the amide (over-all yield, 80 per cent); m.p., 176–178°.

$C_{23}H_{29}O_4N_3$ (411.5). Calculated, N 10.2; found, N 10.1

Hydrogenolysis gave the dipeptide amide acetate in a yield of 53 per cent.

$C_{17}H_{27}O_4N_3$ (337.4). Calculated, N 12.5; found, N 12.3

L-*Leucyl-*L-*phenylalanine Ethyl Ester Hydrochloride*—Hydrogenolysis of the oily carbobenzoxy compound gave the desired product in an over-all yield of 45 per cent; $[\alpha]_D^{22}$ −3.8° (2 per cent in water).

$C_{17}H_{27}O_3N_2Cl$ (342.9). Calculated, N 8.2; found, N 8.2

D-*Leucyl*-L-*phenylalanine Ethyl Ester Hydrochloride*—The carbobenzoxy dipeptide ester was obtained in crystalline form (yield, 46 per cent); m.p., 114–115°.

$C_{23}H_{22}O_5N_2$ (440.5). Calculated, N 6.4; found, N 6.4

Hydrogenolysis gave the dipeptide ester hydrochloride in a yield of 90 per cent; $[\alpha]_D^{22}$ −43.3° (2 per cent in water).

$C_{17}H_{27}O_3N_2Cl$ (342.9). Calculated, N 8.2; found, N 8.1

L-*Lysyl*-L-*tyrosinamide Dihydrobromide*—The dicarbobenzoxy dipeptide ester was obtained as a product melting at 106–107°; yield, 73 per cent.

$C_{33}H_{39}O_8N_3$ (605.7). Calculated, N 6.9; found, N 7.1

The ester was converted nearly quantitatively to the amide; m.p., 183° (19).

$C_{31}H_{36}O_7N_4$ (576.7). Calculated, N 9.7; found, N 9.5

The decarbobenzoxylation was effected by treatment with HBr-acetic acid (20); yield, 92 per cent; $[\alpha]_D^{15}$ +17.3° (2 per cent in water).

$C_{15}H_{25}O_3N_4Br_2$ (470.2). Calculated, N 11.9; found, N 11.6

L-*Lysyl*-L-*tyrosine Ethyl Ester Dihydrochloride*—Hydrogenolysis of the carbobenzoxy compound gave this substance in 97 per cent yield; $[\alpha]_D^{22}$ +16.9° (2 per cent in water).

$C_{17}H_{29}O_4N_3Cl_2$ (410.3). Calculated, N 10.2; found, N 10.0

L-*Lysyl*-L-*phenylalaninamide Dihydrochloride*—The dicarbobenzoxy dipeptide ester was obtained in a yield of 65 per cent; m.p., 131°.

$C_{33}H_{39}O_7N_3$ (589.7). Calculated, N 7.1; found, N 7.2

The corresponding amide was obtained in a yield of 95 per cent; m.p., 170°.

$C_{31}H_{36}O_6N_4$ (560.7). Calculated, N 10.0; found, N 9.7

Hydrogenolysis gave the desired dipeptide amide dihydrochloride in a yield of 89 per cent; $[\alpha]_D^{22}$ +38.0° (2 per cent in water).

$C_{15}H_{26}O_2N_4Cl_2$ (365.3). Calculated, N 15.3; found, N 15.1

L-*Lysyl*-L-*phenylalanine Ethyl Ester Dihydrochloride*—Hydrogenolysis of the carbobenzoxy compound gave this product in 98 per cent yield; $[\alpha]_D^{22}$ +14.5° (2 per cent in water).

$C_{17}H_{29}O_3N_3Cl_2$ (394.3). Calculated, N 10.6; found, N 10.6

ε-*Acetyl*-L-*lysyl*-L-*tyrosinamide Hydrochloride*—ε-Acetyl-L-lysine (21) was carbobenzoxylated in the usual manner to yield an oily product. The

coupling reaction gave the desired compound in 78 per cent yield; m.p., 165°.

$$C_{27}H_{35}O_7N_3 \ (513.6). \quad \text{Calculated, N } 8.2; \text{ found, N } 7.9$$

The acylated dipeptide ester was converted to the amide with a yield of 93 per cent; m.p., 235°.

$$C_{25}H_{32}O_6N_4 \ (484.6). \quad \text{Calculated, N } 11.6; \text{ found, N } 11.3$$

Hydrogenolysis gave the desired product in 97 per cent yield; $[\alpha]_D^{22} +17.5°$ (1 per cent in water).

$$C_{17}H_{27}O_4N_4Cl \ (386.9). \quad \text{Calculated, N } 14.4; \text{ found, N } 14.2$$

ε-Acetyl-L-lysyl-L-tyrosine Ethyl Ester Hydrochloride—Hydrogenolysis of the carbobenzoxy compound gave this product with a yield of 85 per cent; $[\alpha]_D^{22} +13.2°$ (1 per cent in water).

$$C_{19}H_{30}O_5N_3Cl \ (415.9). \quad \text{Calculated, N } 10.1; \text{ found, N } 9.8$$

ε-Acetyl-L-lysyl-L-phenylalaninamide Hydrochloride—The coupling product was obtained in a yield of 76 per cent; m.p., 157°.

$$C_{27}H_{35}O_6N_3 \ (497.6). \quad \text{Calculated, N } 8.4; \text{ found, N } 8.2$$

The corresponding amide was obtained in a yield of 95 per cent; m.p., 236°.

$$C_{25}H_{32}O_5N_4 \ (468.6). \quad \text{Calculated, N } 11.9; \text{ found, N } 11.7$$

Hydrogenolysis gave the dipeptide amide hydrochloride; yield, 93 per cent; $[\alpha]_D^{22} +28.0°$ (1 per cent in water).

$$C_{17}H_{27}O_3N_4Cl \ (370.9). \quad \text{Calculated, N } 15.1; \text{ found, N } 14.9$$

ε-Acetyl-L-lysyl-L-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of the carbobenzoxy compound gave the acetyl dipeptide ester in 75 per cent yield; $[\alpha]_D^{22} +12.6°$ (2 per cent in water).

$$C_{19}H_{30}O_4N_3Cl \ (399.9). \quad \text{Calculated, N } 10.5; \text{ found, N } 10.5$$

When 2 ml. of a 0.05 M solution of this substance were kept at pH 7.5 and 37° for 24 hours, 8.7 mg. of a crystalline compound separated; m.p., 238–239°. Its nitrogen analysis agrees with that of the diketopiperazine.

$$C_{17}H_{23}O_3N_3 \ (317.4). \quad \text{Calculated, N } 13.2; \text{ found, N } 12.9$$

ε-Benzoyl-L-lysyl-L-tyrosinamide Hydrochloride—ε-Benzoyl-L-lysine was prepared by the method of Kurtz (22) and carbobenzoxylated in the usual manner to yield an oily product (yield, 96 per cent). The coupling product also could not be crystallized and was converted to the amide (over-all yield, 54 per cent); m.p., 215–216°.

$$C_{30}H_{34}O_6N_4 \ (546.6). \quad \text{Calculated, N } 10.2; \text{ found, N } 10.0$$

Hydrogenolysis gave the benzoyl dipeptide ester as a non-crystalline solid in a yield of 86 per cent; $[\alpha]_D^{22}$ +32.6° (1 per cent in water). The analysis was not satisfactory.

$C_{22}H_{29}O_4N_4Cl$ (449.0). Calculated, N 12.5; found, N 11.8

ε-Benzoyl-L-lysyl-L-tyrosine Ethyl Ester Hydrochloride—Hydrogenolysis of the carbobenzoxy compound gave the product in a yield of 95 per cent; $[\alpha]_D^{22}$ +24.3° (1 per cent in water).

$C_{24}H_{32}O_5N_3Cl$ (478.0). Calculated, N 8.8; found, N 8.6

ε-Benzoyl-L-lysyl-L-phenylalaninamide Hydrochloride—The coupling product was obtained in a yield of 69 per cent; m.p., 140°.

$C_{32}H_{37}O_6N_3$ (559.7). Calculated, N 7.5; found, N 7.5

The amide was prepared with a yield of 82 per cent; m.p., 210°.

$C_{30}H_{34}O_6N_4$ (530.6). Calculated, N 10.6; found, N 10.6

Hydrogenolysis gave the desired product as a non-crystalline solid; yield, 88 per cent; $[\alpha]_D^{22}$ +30.5° (1 per cent in water).

$C_{22}H_{29}O_2N_4Cl$ (433.0). Calculated, N 12.9; found, N 12.6

ε-Benzoyl-L-lysyl-L-phenylalanine Ethyl Ester Hydrochloride—Hydrogenolysis of the carbobenzoxy compound gave the product in a yield of 93 per cent; $[\alpha]_D^{22}$ +21.3° (1 per cent in water).

$C_{24}H_{32}O_4N_3Cl$ (462.0). Calculated, N 9.1; found, N 8.8

Acetyl-L-phenylalanine Ethyl Ester—This substance was prepared by the treatment of acetyl-L-phenylalanine with HCl in absolute ethanol. Yield, 85 per cent; m.p., 93–94°.

$C_{13}H_{17}O_3N$ (235.3). Calculated, N 6.0; found, N 5.9

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SUMMARY

A study of the action of purified beef spleen cathepsin C on a series of dipeptide amides and dipeptide esters at pH 5 has shown that the rate of hydrolysis of the amide or ester bond depends on the structure and configuration of both amino acid residues present in the compound. The presence of D-amino acid residues in the substrate renders the sensitive bond more resistant to hydrolysis by cathepsin C. The rate of action of the enzyme is also decreased by the presence of large side chain groups (isobutyl, ε-acetylamino-n-butyl) in the N-terminal L-amino acid residue of the substrate. Although methylation of the terminal α-amino groups of a

substrate such as glycyl-L-tyrosinamide diminishes the rate of hydrolysis, comparable derivatives of L-prolyl dipeptides are hydrolyzed rapidly. The effect of structural changes in substrates of cathepsin is evident also at pH 7.5, where transamidation reactions can occur. The substances resistant to hydrolysis at pH 5 do not participate as substrates in polymerization reactions at pH 7.5. The L-prolyl dipeptide esters and amides, though hydrolyzed at pH 7.5, do not form polymers, since these substrates do not appear to be active as replacement agents.

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STUDIES ON CARBOHYDRATE METABOLISM IN RAT LIVER SLICES

VI. HORMONAL FACTORS INFLUENCING GLUCOSE-6-PHOSPHATASE*

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In any consideration of carbohydrate metabolism of the liver, glucose-6-phosphate plays an important rôle. Glucose must first be phosphorylated to glucose-6-phosphate in order to enter the glycolytic pathway to form glycogen and fat for storage, or to be burned for energy. On the other hand, liver phosphatase must split glucose-6-phosphate into glucose and inorganic phosphate before stored carbohydrate, or that arising from gluconeogenesis, can be supplied to the blood as glucose (1). Glucose-6-phosphate is hydrolyzed by an organic phosphatase that is associated with the microsome fraction of liver cells (2). This enzyme appears to have a high degree of specificity for glucose-6-phosphate and differs in several respects from the less specific acid and alkaline phosphatases. The activity of this enzyme has been assayed (*a*) by measuring the release of inorganic phosphate from glucose-6-phosphate (G-6-P) (3) and (*b*) by measuring manometrically the glucose released from G-6-P when this substrate was incubated with liver homogenates and glucose oxidase (4).

There is ample reason to believe that the activity of liver glucose-6-phosphatase changes markedly with various states of carbohydrate metabolism. Extremely low glucose-6-phosphatase activity has been found in cases of von Gierke's (glycogen storage) disease (5) in which the blood sugar is low and liver glycogen is very high. Nemeth (6) has recently demonstrated that the glycogen content of fetal liver is related to liver glucose-6-phosphatase activity. On the other hand, glucose-6-phosphatase activity greater than normal has been found in the liver of fasting and alloxan-diabetic rats (4, 7, 8).

These observations have led us to investigate the changes in liver glucose-6-phosphatase activity in animals after the injection or removal of hormones known to influence carbohydrate metabolism.

EXPERIMENTAL

Preparation of Animals—Adult male albino rats, weighing 200 to 300 gm., of the Wistar strain, maintained on Purina laboratory chow, were used unless otherwise specified. Diabetes was produced by the intravenous injection of alloxan (40 mg. per kilo). Only animals which had blood sugar greater than 300 mg. per cent and in which at least 15 days had elapsed after injection of alloxan were used. Bilateral adrenalectomy was performed under ether anesthesia and the animals were used 4 to 6 days after operation. Adrenalectomized rats were maintained on 1 per cent NaCl *ad libitum*. Hypophysectomized rats (Sprague-Dawley) were purchased from the Hormone Assay Laboratories. Normal rats from the same source were maintained under conditions identical to those used for the hypophysectomized animals. These rats were maintained on 5 per cent sucrose solution and Purina laboratory chow *ad libitum*. Seminal vesicle size was taken as evidence of completeness of hypophysectomy.

Rats receiving insulin were injected subcutaneously with 5 units of protamine Zn insulin at 12 hour intervals during the course of treatment. An aqueous suspension of microcrystals of 17-hydroxycorticosterone was injected subcutaneously (5 mg. per 12 hours) when adrenal cortical hormone was studied over a 48 hour period. For shorter time studies, a solution of this hormone in propylene glycol was injected subcutaneously (5 mg. per 12 hours).

Medium and Substrates—In liver slice experiments the animals were sacrificed under Amytal anesthesia and the liver was removed and sliced with a Stadie slicer. Liver slices were incubated in a medium containing, in millimoles per liter, $K^+ = 70$, $Mg^{++} = 20$, $Ca^{++} = 10$, $HCO_3^- = 40$, $Cl^- = 90$. The solutions were equilibrated with 5 per cent CO_2-95 per cent O_2, giving a pH in the presence of liver slices varying between 7.4 and 7.5. Potassium pyruvate-2-C^{14}, 40 mmoles per liter, was used as added substrate.

Methods—Liver glucose-6-phosphatase was assayed by the phosphate release method previously described (4). 0.5 gm. of liver was homogenized in 6 ml. of distilled water and 0.1 ml. of this solution was incubated for 30 minutes at 30° with 0.2 M G-6-P in 0.1 M citrate buffer (pH 6.3), and the release of inorganic phosphate measured by the method of Fiske and Subbarow (9). In the liver slice experiments, the procedures for chemical and isotopic analyses used were the same as those previously described (10).

Results

Glucose-6-phosphatase Activity of Liver Homogenates—The data on glucose-6-phosphatase activity of rat livers under the several experimental conditions are presented in Table I, and for convenience of discussion the mean values of significance are graphically compared in Fig. 1.

By our assay, liver from normal fed male rats had a mean activity of 157, expressed as micromoles of G-6-P hydrolyzed per gm. of wet liver per 30 minutes. This activity was increased to 225 per cent of normal in alloxan diabetes. Adrenalectomy in the normal rat was without effect,

TABLE I

Glucose-6-phosphatase Activity of Rat Liver Homogenates

Glucose-6-phosphatase activity per gm. of liver is expressed as micromoles of G-6-P split per 30 minutes at 30°, while activity per total liver is expressed in micromoles $\times 10^{-3}$ for convenience.

Animals	No. observed	Mean	Standard error	P*
Activity per gm.				
Normal	12	157	12	
Diabetic	12	354	20	<0.01
Fasted normal	13	268	8	<0.01
Fasted diabetic	7	288	26	<0.01
Normal + cortical hormone	6	210	6	<0.01
Normal + insulin	7	107	6	<0.01
Adrenalectomized	5	178	17	>0.05
Adrenalectomized + insulin	6	78	4	<0.01
Diabetic-adrenalectomized	6	172	15	>0.05
Diabetic-adrenalectomized + cortical hormone	6	341	13	<0.01
Normal†	6	145	5	
Hypophysectomized	7	135	2	
Hypophysectomized + insulin 24 hrs.	5	120	1	<0.01‡
Hypophysectomized + insulin 48 hrs.	4	58	2	<0.01‡
Hypophysectomized + cortical hormone	4	157	5	<0.05‡
Activity per total liver				
Normal	6	2.37	0.14	
Diabetic	6	5.25	0.47	<0.01
Fasted normal	7	2.60	0.14	>0.05
Fasted diabetic	4	3.00	0.20	>0.05
Normal + cortical hormone	4	2.51	0.10	>0.05
Normal + insulin	7	1.66	0.14	<0.01
Adrenalectomized	5	1.86	0.10	<0.05
Adrenalectomized + insulin	6	1.14	0.07	<0.01
Diabetic-adrenalectomized	4	2.62	0.17	>0.05
Diabetic-adrenalectomized + cortical hormone	4	3.96	0.17	<0.01

* Calculated P value as compared with normal fed animals.

† Normal rats obtained from the Hormone Assay Laboratories and maintained under the same conditions as the hypophysectomized animals.

‡ Calculated P value as compared with hypophysectomized animals.

but adrenalectomy in the diabetic returned the liver phosphatase activity to normal. That this effect was due to the removal of adrenal cortical hormone was evidenced by the increase in phosphatase in the adrenalectomized-diabetic animals to diabetic levels, 218 per cent of normal, by the injection of cortical hormone over a 48 hour period. Injection of corticoid in the normal resulted in a slight increase in the glucose-6-phosphatase activity (134 per cent). Weber *et al.* have observed a similar increase in phosphatase following administration of cortisone acetate over a 5 day period (11). We have previously found that injection of insulin

[Figure 1: Bar chart. Y-axis labeled μM G-6-P/GM/30MIN with markings at 300 and 150. Bars labeled: NORMAL, DIABETIC, ADX N, ADX D, ADX D+E, N+E, N+I, ADX+I. NO.OBS. 12 12 5 6 6 6 7 6]
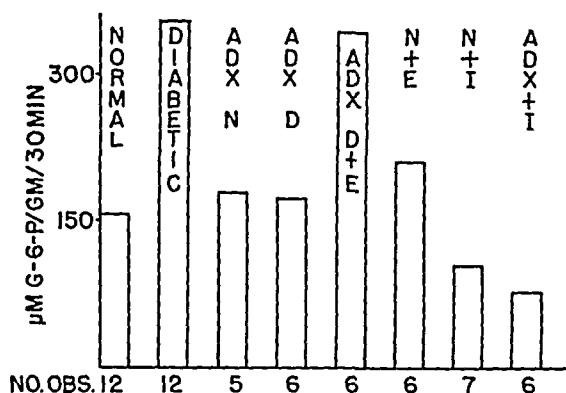
FIG. 1. Changes in rat liver glucose-6-phosphatase with insulin and adrenal cortical hormone. The micromoles of G-6-P split per gm. of wet liver per 30 minutes at 30° are plotted for the various conditions studied. Changes with cortisone were measured 48 hours after subcutaneous injection of a microcrystalline suspension of the hormone. Insulin effects were measured following 24 hours treatment with protamine Zn insulin. Adx N = adrenalectomized animal; adx D = adrenalectomized-diabetic; Adx D + E = adrenalectomized-diabetic animals receiving cortisone; N + E = normal rats receiving cortisone; N + I = normal rats receiving insulin; Adx + I = adrenalectomized rats treated with insulin.

into diabetic rats over a 48 hour period will return glucose-6-phosphatase activity per gm. of liver toward normal (4). Injection of insulin in the normal animals caused phosphatase activity to decrease to 68 per cent of normal, while injection of insulin into adrenalectomized rats resulted in a 50 per cent decrease in phosphatase activity.

Livers of hypophysectomized rats have an essentially normal glucose-6-phosphatase activity. Injection of insulin in these animals over a 48 hour period caused phosphatase activity to be reduced to 37 per cent of the normal value. Administration of cortical hormone caused a slight (116 per cent) increase in phosphatase activity.

In the lower part of Table I, glucose-6-phosphatase activity of total liver mass has been calculated. Phosphatase activity per total liver (activity per gm. of liver × total liver weight) paralleled the activity per

gm., except in the fasted animals and cortical hormone-treated normal animals. In both of these latter cases, a reduction in liver mass occurred approximately equivalent to the increase in glucose-6-phosphatase concentrations.

TABLE II

Glucose-6-phosphatase Activity of Rat Liver Microsomes

Animal	No observed	$\dfrac{\mu\text{mole G-6-P}}{\text{Mg. N}}$	Standard error
Normal	4	13.7	±0.8
Diabetic	4	22.2	±0.7
Adrenalectomized-diabetic	3	11.2	±0.7
Hypophysectomized	4	11.0	±0.3
" + insulin, 24 hrs	5	8.4	±0.45
" + corticoid	4	13.4	±1.7
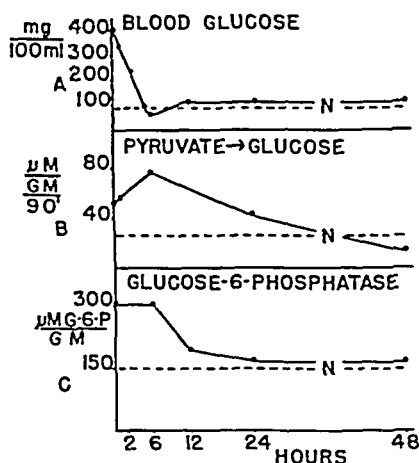
FIG. 2. Biochemical sequence of events after insulin administration to diabetic rats. Curve A, blood glucose in mg. per 100 ml.; Curve B, values expressed in micromoles of pyruvate-2-C^{14} incorporated into glucose per gm. of liver per 90 minutes; Curve C, rat liver glucose-6-phosphatase activity as micromoles of G-6-P split per gm. per 30 minutes at 30°. N, mean normal value.

Glucose-6-phosphatase Activity of Liver Microsomes—Since glucose-6-phosphatase is found in the microsome subcellular fraction, livers from normal, diabetic, adrenalectomized-diabetic, and hypophysectomized rats treated with insulin were homogenized in cold distilled water and the microsomes collected by differential centrifugation (12). 80 to 95 per cent of the activity of the whole homogenate was recovered in the microsome fraction. The activities of these fractions expressed in terms of G-6-P hy-

drolyzed per mg. of microsome N per 30 minutes are given in Table II. Nitrogen was determined by the micro-Kjeldahl method. The glucose-6-phosphatase activities found in the microsome fractions paralleled those found in whole homogenates for the various animal preparations tested.

TABLE III

Incorporation of Carbon from Pyruvate into Glucose, Glycogen, and Fatty Acids by Liver Slices at Varying Time Intervals after Corticoid Administration

All the values are expressed as micromoles per gm. of wet liver per 90 minutes.

Experiment No.	Duration of corticoid administration	Pyruvate utilized	Glycogen		Glucose		Pyruvate to fatty acid
			Net change	Pyruvate to	Net change	Pyruvate to	
Normal							
	hrs.						
1	0	270	−64	17.3	58	9	41
2	0	287	−44	12.4	82	23	46
3	0	250		9.4	81	16	50
Diabetic-adrenalectomized							
1	0	242	−55	8.9	62	14	15
2	0	222	−72	15.0	67	12	51
3	0	241	−164	13.6	62	29	16
4	0	233	−217	11.2	62	25	29
5	0	276	−67	21.0	67	31	30
6	2	282	−102	9.3	69	34	12
7	2	268	−106	9.5	51	29	8
8	6	287	−76	14.6	92	66	10
9	6	250	−71	14.2	106	56	7
10	12	234	−42	7.5	115	55	3
11	12	214	−25	12.0	91	46	3
12	24	230	−63	5.0	89	44	4
13	24	246	−47	7.4	82		5
14	24	264		5.0	105	52	5
15	24	270	−23	4.7	142	47	6
16	24	222		2.9	127	40	6
17	48	350	−80	9.2	136	64	4
18	48	294	−19	8.3	120	45	2
19	48	286	−29	7.0	126	45	1
20	48	263	−24	8.5	107	46	5
Diabetic							
1	0	276	−56	4.0	94	52	1
2	0	250	−33	1.6	131	54	4
3	0	281	−40	2.4	82	40	4

Biological Sequence of Events after Insulin Injection—We have extended the previously reported metabolic changes in metabolism of liver slices of alloxan-diabetic rats sacrificed at various times after insulin administration (10) to include glucose-6-phosphatase activities. The experiments reported here (Fig. 2) relate the fall of blood sugar in the animal (Curve A), incorporation of carbon from pyruvate-2-C^{14} into glucose (Curve B), and changes in liver glucose-6-phosphatase (Curve C). There was an immediate response of blood sugar to injected insulin, normal values being reached in 5 hours. Gluconeogenesis (pyruvate to glucose) continued at diabetic
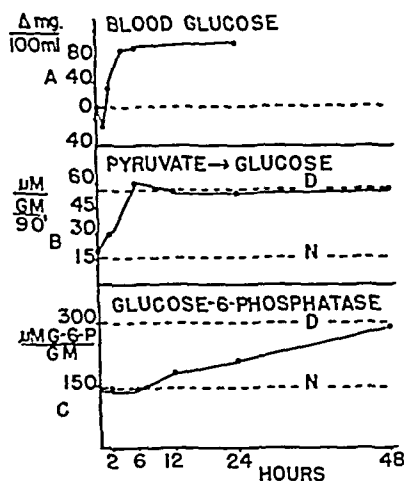

FIG. 3. Biochemical sequence of events after administration of adrenal cortical hormone (5 mg. per 12 hours) to adrenalectomized-diabetic rats. Curve A, change in blood glucose from time zero in mg. per 100 ml. Curve B, values expressed in micromoles of pyruvate-2-C^{14} incorporated into glucose per gm. of liver per 90 minutes. Curve C, rat liver glucose-6-phosphatase activity as micromoles of G-6-P split per gm. per 30 minutes at 30°. N, mean normal value; D, mean diabetic value.

rates during this period and was returned to normal only after 24 to 48 hours of insulin therapy. Phosphatase activity per gm. of liver likewise remained at diabetic levels for 6 hours after administration of insulin but had decreased after 12 hours.

Biological Sequence of Events after Adrenal Cortical Hormone Injection—Since we had found that 48 hours after administration of cortical hormone to adrenalectomized-diabetic rats increased the glucose-6-phosphatase activity from normal to diabetic values, changes in phosphatase activity and pyruvate-2-C^{14} metabolism were followed with time after cortical hormone administration, as in the time-insulin studies. The data on the metabolism of pyruvate-2-C^{14} by liver slices from such animals are presented in Table III, and mean values are plotted against time in Fig. 3.

The incorporation of pyruvate carbon into glucose, glycogen, and fatty acids was essentially normal in the adrenalectomized-diabetic liver. Administration of cortical hormone *in vivo* returns this metabolism to diabetic levels. Cortical hormone caused an increase in pyruvate conversion to glucose within 2 hours; diabetic values were reached in 6 hours (Curve B). The increase in blood glucose (Curve A) parallels the increase in gluconeogenesis shown in Curve B. Liver glucose-6-phosphatase activity (Curve C) was not increased during the first 6 hours after administration of cortical hormone; it was slightly increased at 12 hours and had reached diabetic levels only after 48 hours. Pyruvate conversion to glycogen was not materially decreased until more than 12 hours had elapsed, but pyruvate conversion to fatty acids was appreciably decreased in 2 hours and had reached diabetic values in 12 hours after corticoid administration (Table III).

DISCUSSION

Experimental studies of the diabetic state have emphasized the existence of a hormone balance, involving the pancreas, hypophysis, and adrenal cortex, that participates in the regulation of carbohydrate metabolism in the intact animal. The classical experiments of Houssay (13) and Long and Lukens (14) clearly established the interdependence of these hormones in regulating carbohydrate metabolism. The results of the present study on glucose-6-phosphatase are consistent with these concepts. Administration of adrenal cortical hormone resulted in a greater increase in glucose-6-phosphatase activity in the adrenalectomized-diabetic animals than it did in normal rats. On the other hand, administration of insulin to adrenalectomized animals resulted in a greater decrease in glucose-6-phosphatase activity in these animals than it did in normal animals.

Glucose Production—An attempt has been made to correlate glucose-6-phosphatase activity of the liver with metabolism of liver slices *in vitro* incubated in a medium of intracellular cationic composition. Previous experiments in this laboratory (15) have compared the metabolism of glucose-U-C^{14} and pyruvate-2-C^{14} by liver slices from normal, diabetic, adrenalectomized, and adrenalectomized-diabetic rats. Glucose utilization was reduced in the diabetic and diabetic-adrenalectomized animals. Thus, utilization of glucose appears to be under the control of insulin alone. Glucose production by the liver slice seems to be correlated with tissue glucose-6-phosphatase activity. It was previously shown (15) that glucose production is normal in the adrenalectomized and adrenalectomized-diabetic liver and increased over 2-fold in the diabetic liver. In the present study, it has been found that administration of cortical hormone to adrenalectomized-diabetic rats led to increased gluconeogenesis which pre-

ceded by several hours the increase in liver glucose-6-phosphatase. Thus it seems unlikely that an increased glucose production is the direct result of an increased glucose-6-phosphatase activity in the diabetic animal.

Glycogen Synthesis—The relation of liver glucose-6-phosphatase to glycogen synthesis is also of interest. Sutherland and Cori (16) have demonstrated that the hyperglycemic effects of glucagon and epinephrine are not attributable to this enzyme. Weber and Cantero (7) have suggested that the low liver glycogen found in fasting rats is the result of the increased phosphatase activity observed. In the fetal guinea pig liver, Nemeth (6) has observed a striking correlation between glucose-6-phosphatase and glycogen content. Glycogen in the fetal liver rises steadily during the last few days of gestation and during this period glucose-6-phosphatase activity is virtually absent. At parturition the phosphatase activity increases rapidly and liver glycogen falls off correspondingly. Cori and Cori (5) were the first to suggest that glycogen storage in the liver is due to low activity of this enzyme.

In the present study attempts have been made to relate the per cent of G-6-P converted to glycogen to phosphatase activity. This figure has been derived experimentally from the ratio, pyruvate to glycogen over pyruvate to glucose plus glycogen. This ratio, with corresponding glucose-6-phosphatase activities, has been calculated from data on liver slice studies on adrenalectomized-diabetic animals receiving adrenal cortical hormone (Table IV). It is apparent that, in these rats receiving corticoid, the per cent of G-6-P to glycogen decreased as the glucose-6-phosphatase activity increased. It is, therefore, not unreasonable to expect that glucose-6-phosphatase activity should regulate the relative per cent of G-6-P molecules converted to glucose and glycogen.

Blood Glucose—There appears to be no direct relation of glucose-6-phosphatase activity to blood glucose levels. In experiments with intact animals, phosphatase activity per total liver should be considered rather than activity per gm. of liver. It is evident, however, that blood glucose values may change markedly without any appreciable effect on total liver glucose-6-phosphatase activity. The decrease in blood glucose observed in the diabetic after insulin administration is evidently the result of the immediate action of insulin on peripheral utilization of glucose. Certainly, liver gluconeogenesis (pyruvate conversion to glucose) and glucose-6-phosphatase have not diminished during this period of fall of blood sugar. The blood glucose increase observed in adrenalectomized-diabetic rats injected with cortical hormone is accompanied by an increase in pyruvate to glucose in the liver, but not by an increase in liver phosphatase. The best argument against glucose-6-phosphatase being the determining factor in the regulation of blood sugar concentration is the observation that fasting

normal, fasting diabetic, and fed normal rats all have approximately the
same total liver glucose-6-phosphatase (Table I).   Yet, the fasting normal
will have a blood sugar of 60 mg. per cent, the diabetic 250 mg. per cent,
and the fed normal 100 mg. per cent.

Fatty Acid Synthesis—The synthesis of long chain fatty acids by liver
tissue incubated *in vitro* with acetate, pyruvate, fructose, and glucose
(10, 17–19) is known to be diminished in fasting and alloxan-diabetic rats.
It has been proposed that the effects of fasting and alloxan diabetes upon
lipogenesis may perhaps be ascribed to a diminished availability of intra-

TABLE IV

Relation of Liver Glucose-6-phosphatase to Glycogen Formation

Glucose-6-phosphatase activity is expressed as micromoles of G-6-P split per gm.
of liver per 30 minutes.   Per cent G-6-P to glycogen is the ratio, pyruvate-glycogen
to pyruvate-glucose plus glycogen measured in rat liver slices during a 90 minute
incubation.

Rat	Time	Per cent G-6-P to glycogen	Glucose-6-phosphatase
	hrs.		
Normal...........................		50	157
Diabetic.........................		12	354
Adrenalectomized-diabetic.................		35	150
Adrenalectomized, normal.................		47	178
Adrenalectomized-diabetic + 17-hydroxy-corticosterone........................	2	24	155
" "	6	23	162
" "	12	17	240
" "	24	10	246
" "	48	15	310

cellular substrates for glycolysis (20).   Others have described the dimin-
ished lipogenesis of the diabetic liver as a second metabolic lesion (21).
It is evident that adrenal cortical hormones decrease (21) and that insulin
increases (17) fatty acid synthesis in the liver.   Brady *et al.* (22) have
demonstrated that fatty acid synthesis by liver slices from depancreatized
cats is returned toward normal by adrenalectomy or hypophysectomy.
In the present study with alloxan-diabetic rats, it has been confirmed that
adrenalectomy does restore fatty acid synthesis.   Liver glucose-6-phos-
phatase is also returned to normal in these animals.   The question arises
as to whether or not glucose-6-phosphatase activity might influence the
concentration of glycolytic intermediates and by this means also influence
fatty acid synthesis.   It seems unlikely that this is an important factor,
since, in experiments in which adrenal cortical hormone was administered

to adrenalectomized-diabetic rats, diminished fatty acid synthesis occurred several hours prior to any observed changes in glucose-6-phosphatase.

SUMMARY

1. The biochemical sequence of events has been determined in rat liver slices after injection of insulin and adrenal cortical hormone to diabetic and adrenalectomized-diabetic rats.

2. The activity of glucose-6-phosphatase per gm. of wet liver was found to be increased over normal in fasting and alloxan-diabetic rats. Diabetic-adrenalectomized rats had normal liver glucose-6-phosphatase activity. Injection of insulin *in vivo* decreased liver glucose-6-phosphatase, while adrenal cortical hormones tended to increase liver phosphatase.

3. The time required to change the activity of liver glucose-6-phosphatase was determined in diabetic rats injected with insulin and adrenalectomized-diabetic rats injected with adrenal cortical hormones. 6 to 12 hours were required before hormone-induced changes in liver glucose-6-phosphatase activity were observed.

4. The rôle of glucose-6-phosphatase in diabetes has been examined. It was found that the increased gluconeogenesis and decreased fatty acid synthesis characteristic of diabetic liver metabolism precede any observed changes in liver glucose-6-phosphatase activity.

5. Adrenal cortical hormones appeared to act within 2 hours of injection to alter pyruvate metabolism in liver slices from adrenalectomized-diabetic rats. This is contrasted with the observed 12 to 24 hours delay in changes in pyruvate metabolism, following insulin injection into alloxan-diabetic rats.

6. Glucose-6-phosphatase activity appeared to influence the relative per cent of glucose-6-phosphate converted to glucose and glycogen.

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THE INTRACELLULAR DISTRIBUTION OF PENTOSE CYCLE ACTIVITY IN RABBIT KIDNEY AND LIVER*

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Horecker (1) has demonstrated the occurrence of a cyclic mechanism in yeast and animal tissues which can account for the oxidation of carbohydrate to carbon dioxide and water without participation of triosephosphate dehydrogenase or the enzymes of the tricarboxylic acid cycle. The term "pentose cycle" has been suggested (2) for this series of reactions previously called the "shunt" or "oxidative pathway," since the reactions occur in a cyclic manner and involve pentoses as key compounds. Knowledge of the presence of this cycle has been extended to include plants (3), bacteria (4), insects (5), and the wheat smut fungus (6). This cycle might function as an energy source and also as a means by which ribose, an important constituent of nucleic acids and certain coenzymes, can be synthesized or catabolized.

Several workers (7, 8) have demonstrated the nearly exclusive location within the mitochondria of many oxidative enzymes, particularly those involved in the tricarboxylic acid cycle, various electron carrier systems, and oxidative phosphorylation. The glycolytic enzymes for the anaerobic conversion of carbohydrate have been shown to be localized in the soluble portions of the cell (9). Since the reactions of the pentose cycle may conceivably be associated with either or several of these activities, their behavior in the centrifugal field seemed of interest.

Several experimenters (10–14) have compared the quantitative contribution of the pentose cycle and the glycolytic scheme (together with the Krebs cycle) to the oxidation of carbohydrate. Organisms which contain little or no Krebs cycle activity may obtain much of their energy and assimilate carbon by such a mechanism, as suggested by Scott and Cohen (15) for

Escherichia coli. In this organism the rate of assimilation of glucose by the pentose cycle is considered similar to the combined rate of assimilation by glycolysis and the Krebs cycle.

Methods and Materials

Preparation of Subcellular Fractions—The fresh liver (divested of the gallbladder) or kidneys from decapitated rabbits were removed and packed in ice. The medulla of the kidney was removed and the cortical material used for the fractionation of kidney homogenates. The livers or kidneys were then cut into small pieces and homogenized in 6 volumes of a solution containing 0.001 M sodium Versenate and either (a) 0.25 M sucrose or (b) 0.001 M citrate in 0.9 per cent KCl. Homogenizing was usually carried out in a Waring blendor for 30 seconds at half speed. The KCl preparations were generally used, since large amounts of sucrose greatly interfered in the colorimetric methods used for the pentose cycle sugars. The pH was constantly adjusted with 1 N KOH to 7.0 to 7.5 during the blending operation.
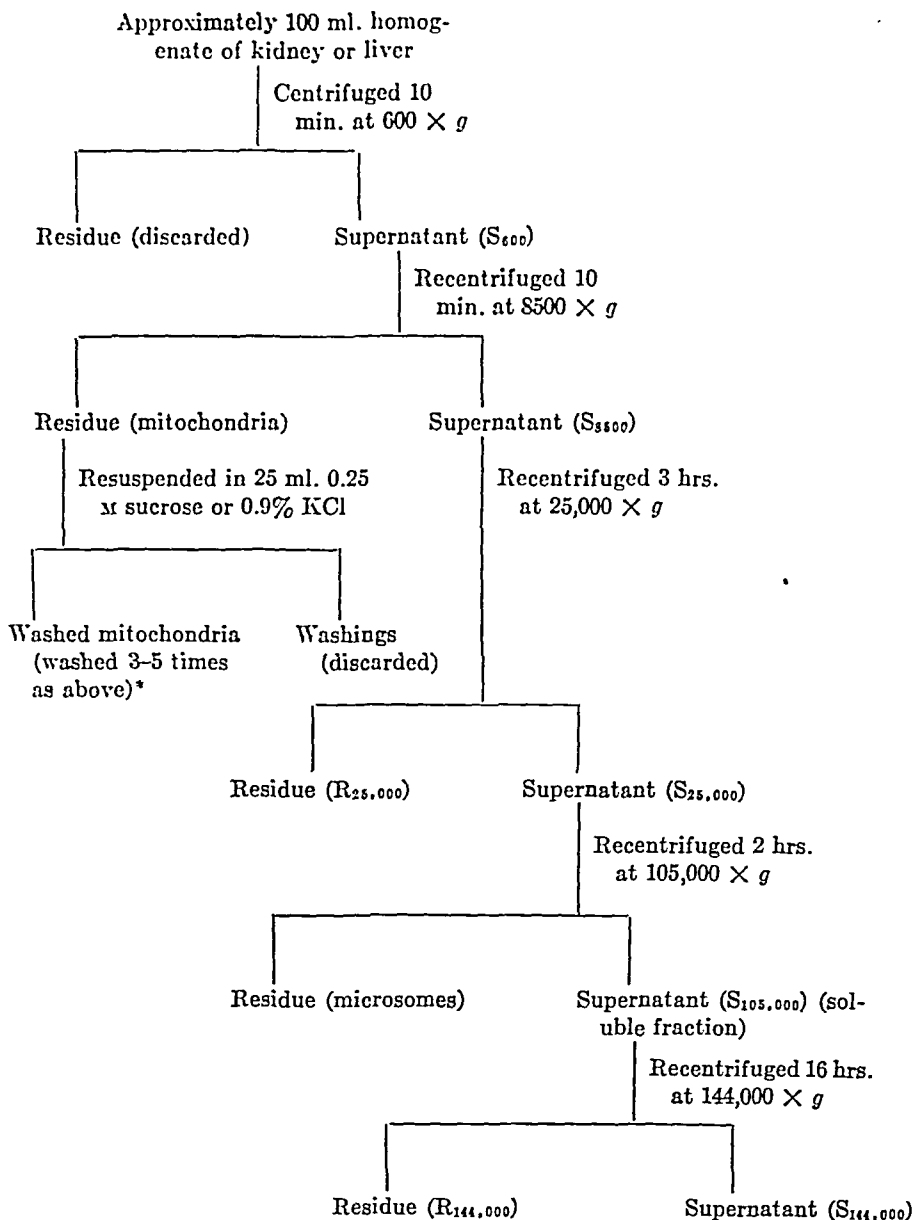
The material was then centrifuged according to the scheme presented in Diagram 1. An International refrigerated centrifuge with a high speed attachment was used for centrifugal forces up to $25,000 \times g$. A Spinco model L ultracentrifuge was used for higher speeds (for speeds up to $105,000 \times g$ the No. 30 head, and for speeds up to $144,000 \times g$ the No. 40.3 head). All operations were performed at 0–$5°$. For the kidney experiments, the mitochondrial fraction was washed three to five times with the appropriate isotonic solution before use. The citrate-oxidizing capacity was only slightly impaired, even over the 6 hour period required for centrifuging. Kidney preparations with sucrose and KCl showed similar activity, but liver mitochondria seemed more stable when prepared in sucrose, as suggested by Hogeboom *et al.* (17).

Rat livers were also tested. For these experiments, the tissues were homogenized in a Potter-Elvehjem glass homogenizer.

A Warburg apparatus was employed for the manometric experiments. Gassing was performed with a conventional manifold. A Bausch and Lomb spectronic 20 spectrophotometer was used for the colorimetric assays and a Beckman spectrophotometer for the studies of triphosphopyridine nucleotide reduction. The methods used to follow non-oxidative pentose cycle conversions have been described previously (5, 6).

Materials—The following materials were commercial preparations: glucose-6-phosphate (G-6-P), triphosphopyridine nucleotide (TPN), cytochrome *c*, and adenosine-5'-phosphate, all from the Sigma Chemical Company; ribose-5-phosphate (R-5-P), from the Schwarz Laboratories, Inc.; thiamine pyrophosphate (TPP) from Hoffmann-La Roche, Inc.; and

Diagram 1. Scheme for Fractionation of Rabbit Liver or Kidney by Differential Centrifugation

Approximately 100 ml. homogenate of kidney or liver

Centrifuged 10 min. at 600 × g

Residue (discarded)   Supernatant (S_{600})

Recentrifuged 10 min. at 8500 × g

Residue (mitochondria)   Supernatant (S_{8500})

Resuspended in 25 ml. 0.25 M sucrose or 0.9% KCl

Recentrifuged 3 hrs. at 25,000 × g

Washed mitochondria (washed 3–5 times as above)*   Washings (discarded)

Residue ($R_{25,000}$)   Supernatant ($S_{25,000}$)

Recentrifuged 2 hrs. at 105,000 × g

Residue (microsomes)   Supernatant ($S_{105,000}$) (soluble fraction)

Recentrifuged 16 hrs. at 144,000 × g

Residue ($R_{144,000}$)   Supernatant ($S_{144,000}$)

* Similar to the preparations of Green *et al.* (16) (with KCl), or of Hogeboom *et al.* (17) (with sucrose).

Armour's coenzyme concentrate from Armour and Company. N-Methyl-phenazine sulfate, sedosan, and disodium Versenate were kindly supplied by Dr. R. H. Burris, Dr. N. E. Tolbert, and Versenes, Inc.; 6-phospho-gluconic acid (6-PGA) was prepared as before (5).
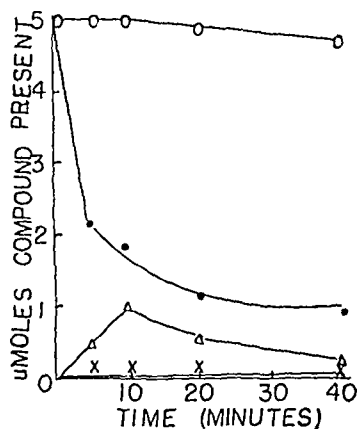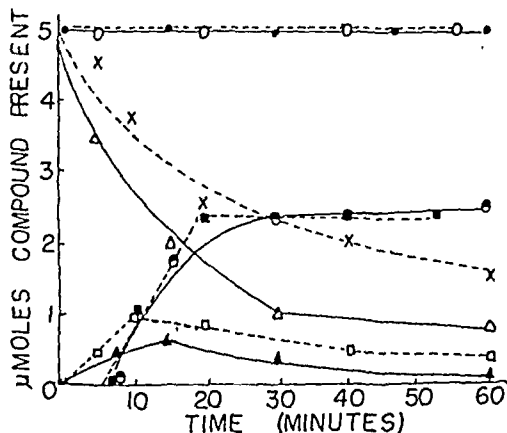

FIG. 1   FIG. 2

FIG. 1. Pentose cycle activity in rabbit liver fractions (non-oxidative). Each tube contained 1 ml. of enzyme, 200 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 8.0, 100 γ of TPP, 5 μmoles of R-5-P. Total volume 7.6 ml.; temperature 37°. 1 ml. aliquots were removed at the times designated and pipetted into 1 ml. of 10 per cent trichloroacetic acid. After centrifugation, 0.5 ml. aliquots were removed for the determination of R-5-P, sedoheptulose, and hexose. The mitochondria were resuspended twice in 0.9 per cent KCl. O, R-5-P (mitochondria); ●, R-5-P (S_{8500}); △, sedoheptulose (S_{8500}); ✕, sedoheptulose (mitochondria).

FIG. 2. Pentose cycle in the S_{8500}, $S_{105,000}$, mitochondrial, and microsomal ($R_{105,000}$) fractions of rabbit kidney (non-oxidative). Additions as for Fig. 1. 1 ml. of mito-chondria contained 4 mg. of protein (washed three times with 0.9 per cent KCl); 1 ml. of S_{8500} contained 8 mg. of protein; 1 ml. of $S_{105,000}$ contained 2.4 mg. of protein; 1 ml. of microsomes contained 6 mg. of protein. ●, R-5-P (mitochondria); O, R-5-P (microsomes); △, R-5-P (S_{8500}); ◐, hexose (S_{8500}); ▲, sedoheptulose (S_{8500}); ✕, R-5-P ($S_{105,000}$); ■, hexose ($S_{105,000}$); □, sedoheptulose ($S_{105,000}$).

Results

Citrate oxidation served as the criterion of mitochondrial activity, and its oxidation was nearly complete. G-6-P and R-5-P were not oxidized by the mitochondria; neither were citrate, G-6-P, and R-5-P by the super-natant fractions (except S_{600} which contained the mitochondria). Recom-bination of S_{8500} and the mitochondria resulted in limited oxidation of these substrates. The lowered activity may be due in part to dilution of the oxidative enzymes. Oxidation of G-6-P and R-5-P proceeded in the S_{600} fraction in the presence of cytochrome c and was enhanced when N-methyl-phenazine sulfate was used as an electron carrier. Limited oxidation of

these substances occurred with the $S_{25,000}$ fraction only in the presence of the artificial electron acceptor.

Intracellular Localization of Non-Oxidative Pentose Cycle Enzymes—The disappearance of R-5-P and formation of sedoheptulose and hexose with increasing time were used as evidence for the occurrence of the pentose cycle in the various cellular fractions. This procedure seemed justified, since the bulk of the pentose could be accounted for as sedoheptulose and hexose.

Fig. 1 illustrates the localization of the pentose cycle enzymes in rabbit liver. These are soluble after centrifugation at 8500 × g, whereas little

TABLE I

Intracellular Localization of Pentose Cycle (Non-Oxidative) in Rabbit Kidney

The reaction tubes contained 1 ml. of enzyme of the following fractions: S_{600}, 3.8 mg. of protein; $S_{25,000}$, 2.4 mg. of protein; $S_{105,000}$, 2.0 mg. of protein; the tubes also contained 200 μmoles of tris(hydroxymethyl)aminomethane buffer (pH 8.0), 100 γ of TPP, 20 μmoles of Mg, and 5 μmoles of R-5-P. Total volume 7.7 ml.; temperature 37°. 1 ml. aliquots were removed at various times and added to 1 ml. of 10 per cent trichloroacetic acid, centrifuged to remove protein, and assayed colorimetrically.

Fraction	Total protein	R-5-P disappearing		Sedoheptulose formed		Hexose formed	
		μmoles per mg. protein in original reaction mixture	Total	μmoles per mg. protein in original reaction mixture	Total	μmoles per mg. protein in original reaction mixture	Total
	mg.		*μmoles*		*μmoles*		*μmoles*
S_{600}	362	1.04	378	0.13	47	0.61	220
$S_{25,000}$	228	1.6	365	0.21	48	1.0	228
$S_{105,000}$	190	1.93	370	0.25	47	1.3	246

or no activity is found in the mitochondria. In kidney, the pentose cycle activity remained soluble after 2 hours of centrifugation at 105,000 × g, as shown in Fig. 2. Similar results were obtained with the $S_{25,000}$ fraction. Neither the mitochondria nor microsomes contained activity.

Quantitative recovery of non-oxidative activity in the cycle was sought next, after centrifugation at 600 × g, 25,000 × g, and 105,000 × g. Aliquots were analyzed after various periods of incubation of enzyme with R-5-P. The results for one incubation period (45 minutes) are reported in Table I. The results at other times were similar. It is apparent that all of the original activity was recovered in the supernatant fluid after centrifugation at 105,000 × g for 2 hours.

In another experiment, the $S_{105,000}$ fraction was centrifuged for 16 hours at 144,000 × g. The red-colored material present in the $S_{105,000}$ fraction

sedimented, leaving a clear colorless solution above it. The colorless layer was carefully removed with a pipette and designated the $S_{144,000}$ fraction. The red layer was removed and designated the $R_{144,000}$ fraction. As shown in Fig. 3, it appears that the pentose cycle is contained in the lower red-colored layer.
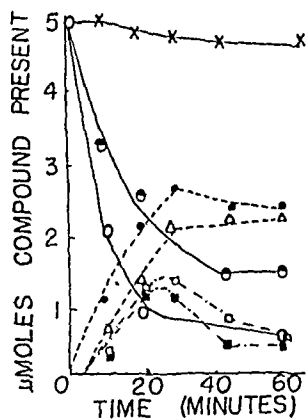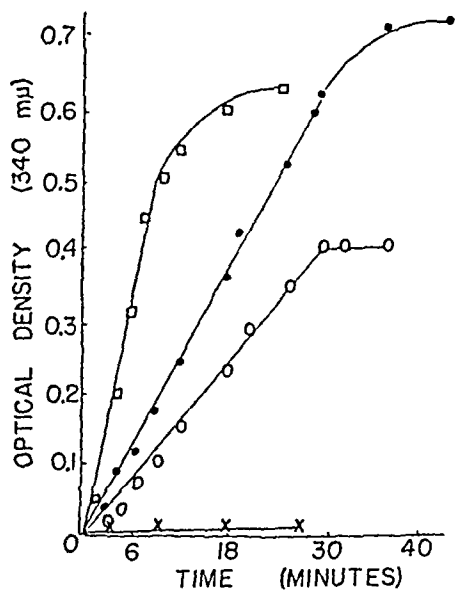

FIG. 3.

FIG. 4.

FIG. 3. Pentose cycle in the $S_{105,000}$, $S_{144,000}$, and $R_{144,000}$ fractions of rabbit kidney (non-oxidative). Additions as for Fig. 1. 1 ml. of $S_{105,000}$ contained 2.2 mg. of protein; 1 ml. of $S_{144,000}$ contained 1 mg. of protein; 1 ml. of $R_{144,000}$ contained 4.7 mg. of protein. Neither hexose nor sedoheptulose was formed with the $S_{144,000}$ fraction. ◓, R-5-P ($S_{105,000}$); ●, hexose ($S_{105,000}$); □, sedoheptulose ($S_{105,000}$); ○, R-5-P ($R_{144,000}$); △, hexose ($R_{144,000}$); ■, sedoheptulose ($R_{144,000}$); ×, R-5-P ($S_{144,000}$).

FIG. 4. Glucose-6-phosphate dehydrogenase in rabbit kidney tissue. The complete system consisted of 20 μmoles of tris(hydroxymethyl)aminomethane buffer, pH 8.0, 10 μmoles of nicotinamide, 0.25 μmole of TPN, 2 μmoles of G-6-P. Total volume 3.5 ml. Appropriate blanks without enzymes, TPN, or substrate were used. The cuvettes for S_{8500} contained 0.8 mg. of protein; for $S_{25,000}$, 0.6 mg. of protein; for $S_{105,000}$, 1.2 mg. of protein; for microsomes ($R_{105,000}$), 3 mg. of protein. The times listed refer to the duration of centrifugation. ○, G-6-P (S_{8500}), 10 minutes; ●, G-6-P ($S_{25,000}$), 3 hours; □, G-6-P ($S_{105,000}$), 2 hours; ×, G-6-P ($R_{105,000}$), 2 hours.

Experiments with rat livers yielded similar results in that no pentose cycle activity was found in the mitochondria, but resided in the $S_{25,000}$ fraction. Higher gravitational fields were not employed.

Glucose-6-phosphate Dehydrogenase and 6-Phosphogluconic Acid Dehydrogenase—The reduction of TPN by G-6-P or 6-PGA was used to localize these enzymes in the various fractions. The soluble nature of G-6-P dehydrogenase is demonstrated in Fig. 4. Equal amounts of dehydrogenase were added to both cuvettes. The turbidity of the S_{600} fraction precluded

accurate recovery data at this point in the centrifugation scheme, and therefore data similar to those obtained for the non-oxidative conversions were not obtained. After centrifugation for 2 hours at 105,000 $\times$ g, no activity was found in the microsomes, but remained instead in the supernatant fluid. The results with 6-PGA were similar.

In another experiment at higher centrifugal forces, *i.e.*, 144,000 $\times$ g for 16 hours, as described previously, a sedimentation of the two dehy-
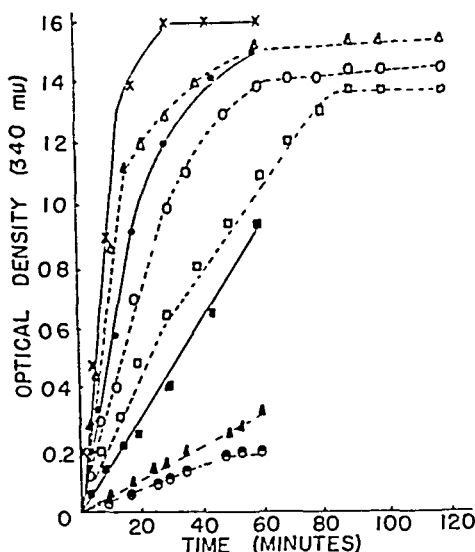

Fig. 5. Reduction of TPN by G-6-P, R-5-P, and 6-PGA in rabbit kidney tissue. Additions same as for Fig. 4. The cuvettes for $S_{105,000}$ contained 1.1 mg. of protein; for $S_{144,000}$, 0.4 mg. of protein; for $R_{144,000}$, 1.0 mg. of protein. $\times$, G-6-P ($S_{105,000}$); $\bullet$, R-5-P ($S_{105,000}$); $\blacksquare$, 6-PGA ($S_{105,000}$); $\triangle$, G-6-P ($R_{144,000}$); $\bigcirc$, R-5-P ($R_{144,000}$); $\square$, 6-PGA ($R_{144,000}$); $\blacktriangle$, G-6-P ($S_{144,000}$); $\ominus$, 6-PGA ($S_{144,000}$).

drogenases occurred as shown in Fig. 5. In addition, TPN was reduced by R-5-P, although at a slower rate than by G-6-P, but more rapidly than by 6-PGA. In the presence of R-5-P and the $S_{144,000}$ enzymes, no reduction of TPN occurred.

The principal remaining step in the pentose cycle comprises the fructose-6-phosphate; fructose-1,6 diphosphate $\rightarrow$ G-6-P conversions. Although these have not been examined directly, the oxidation of R-5-P and its anaerobic conversion to hexose indicate that the hexose isomerases accompany the remainder of the pentose cycle complex. Earlier studies (9) have demonstrated the soluble nature of glycolytic enzymes at somewhat lower centrifugal fields.

DISCUSSION

The apparently similar centrifugal behavior of the entire group of pentose cycle enzymes (including the oxidative steps) suggests the possibility of intimate association among the cycle members, or the presence of a small organelle containing these enzymes. The localization of the pentose cycle apart from the particulate electron carrier systems or the known oxidative phosphorylation apparatus increases the importance of determining how such multienzyme groups may be associated or related to one another.

SUMMARY

The pentose cycle enzymes (non-oxidative) of rabbit kidney and liver tissue have been shown to be soluble after centrifugation at 105,000 $\times$ g for 2 hours, but they sedimented after centrifugation at 144,000 $\times$ g for 16 hours. Glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase behaved in a similar manner.

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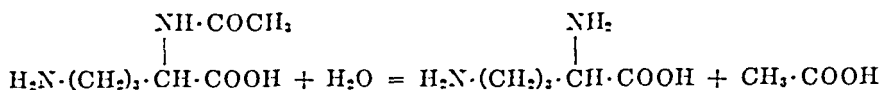
ACETYLORNITHINASE OF ESCHERICHIA COLI: PARTIAL PURIFICATION AND SOME PROPERTIES*

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In *Escherichia coli*, ornithine synthesis has been shown to proceed via glutamate, N-acetylglutamate, N-acetylglutamic γ-semialdehyde, and N^{α}-acetylornithine (1–3). The last step in the formation of ornithine

$$\underset{\substack{|\\H_2N\cdot(CH_2)_3\cdot CH\cdot COOH}}{NH\cdot COCH_3} + H_2O = \underset{\substack{|\\H_2N\cdot(CH_2)_3\cdot CH\cdot COOH}}{NH_2} + CH_3\cdot COOH$$

is catalyzed by the enzyme acetylornithinase (1, 4). The present report is concerned with a characterization of this enzyme.

Materials and Methods

Compounds Used—N^{α}-Acetyl-L-ornithine was synthesized as previously described (5, 3).[1] L-Ornithine monohydrochloride was obtained from the Mann Research Laboratories. Several of the acetylamino acids used, including acetyl-DL-methionine, were supplied by the California Foundation for Biochemical Research.[2]

Organisms—The organisms used are E. coli (ATCC 9637) and ornithine-requiring mutant 160-37 of E. coli (5, 1).

Cultivation—The growth medium used is prepared at 50 × strength as follows. In distilled water (670 ml.) are dissolved, successively, $MgSO_4$·$7H_2O$ (10.0 gm.), citric acid·H_2O (100.0 gm.), K_2HPO_4·anhydrous (500.0 gm.), and $NaNH_4HPO_4$·$4H_2O$ (175.0 gm.), the final volume being about 1.00 liter.[3] On 50-fold dilution with distilled water, the resulting single strength medium, designated E, has a pH of about 7.0; it is sterilized by autoclaving. Medium E is supplemented with dextrose (autoclaved sepa-

[1] Certain laboratory facilities used in this synthesis were generously made available by Dr. J. S. Fruton.

[2] These acetylamino acids were received as a gift. The generosity of the California Foundation for Biochemical Research is gratefully acknowledged.

[3] Mallinckrodt "analytical reagents" were found satisfactory. The 50 × strength medium is stored at room temperature; chloroform (about 1 ml.) may be added as a preservative.

rately) at a concentration of 5 gm. per liter. For strain 160-37 the medium is further supplemented with L-arginine (50 γ per ml.).

The organisms are grown with shaking at 37° in Fernbach flasks containing suitably supplemented medium.

Assay of Enzyme Activity

For acetylornithinase assays, the enzyme is permitted to act under a set of desired conditions, for example "standard conditions" (see below), and the ornithine produced is determined by a new modification of the colorimetric ninhydrin method. The development of this modification was necessitated by the properties of the substrate; at pH values near 5 (cf. (6)), and to a lesser extent in strongly acid solution (cf. (7)), N^α-acetylornithine gives a ninhydrin reaction. In the present method, the ninhydrin is permitted to act at a pH of about 2.5 (measured in the absence of the organic solvent used). Under these conditions N^α-acetylornithine gives but a very slight ninhydrin reaction.

Ninhydrin Reagent—The reagent used is prepared by mixing 2 volumes of a 1 per cent solution of ninhydrin in Methyl Cellosolve with 1 volume of 0.4 M aqueous citric acid.[4]

Determination of Ornithine—The ornithine-containing samples are prepared in 0.1 M aqueous phosphate (pH 7),[5] 1 mM with respect to glutathione. To 0.5 ml. portions of each of the samples are added 1.5 ml. of the ninhydrin reagent. Appropriate standards and blanks (see below) are run along with the samples. The resulting reaction mixtures are heated in a boiling water bath for 10 minutes and then cooled. If ornithine is present, a blue color is produced.[6] To each reaction mixture are added 3 ml. of 0.7 N aqueous sodium hydroxide with immediate stirring.[7] A transient intense yellow color appears even in the absence of ornithine. 20 minutes after addition of the alkali, the final reaction mixtures are read against reference blanks (see below) in a Klett-Summerson colorimeter with the No. 42 filter. Ornithine gives rise to an orange-yellow color which is stable for hours.

[4] The ninhydrin reagent is stored at 3°; it remains usable for at least 1 week.

[5] The phosphate and the citric acid (in the ninhydrin reagent) provide the desired pH.

[6] If the glutathione is omitted from the reaction mixtures, the color yield is lowered. Certain reducing agents other than glutathione also enhance the color yield.

[7] The reasons for adding the alkali are 2-fold: (a) the spectrum of the initially produced blue color is a rather sensitive function of the hydrogen ion concentration, whereas the orange-yellow color obtained on addition of alkali is not, and (b) the alkali tends to dissolve any added protein that may have precipitated under acid conditions. The assay can be carried out even in the presence of coarse débris or whole cells.

A linear color response to ornithine is obtained up to at least 0.3 μmole of ornithine per sample. Absorption maxima occur at 350 and 470 mμ. A Klett-Summerson reading of 160 corresponds to optical densities of 0.495 and 0.320 at 350 and 470 mμ, respectively, as determined in the Beckman spectrophotometer (light path, 1 cm.).

The present modification of the ninhydrin method may be applied to the determination of proline. Certain other amino acids, including lysine, cysteine, and α,ϵ-diaminopimelic acid,[8] also produce colors.

Standard Conditions for Enzyme Assays—Assays are performed in 4 inch test-tubes by incubating potassium phosphate at pH 7 (50 μmoles), N^α-acetylornithine (3 μmoles), glutathione (0.5 μmole), cobaltous chloride (0.1 μmole), and enzyme (usually added last)[9] in aqueous solution (total volume, 0.5 ml.) at 37° for 10 minutes.

Colorimetry—At the end of the incubation period the enzymatic reaction is stopped by the addition of 1.5 ml. of the ninhydrin reagent to each tube, and ornithine is determined as described above. In addition to the samples to be assayed, reference blanks (substrate and enzyme omitted), enzyme blanks (substrate omitted), and substrate blanks (enzyme omitted) are run. Assay results are computed by subtracting the values obtained for the respective enzyme and substrate blanks from the values for the assay samples. Typical blanks as well as the response to ornithine are shown in Table I.[10]

Unit of Enzyme Activity—1 unit of acetylornithinase activity is defined as that amount of enzyme which will catalyze the formation of 0.1 μmole of ornithine under standard conditions.

Protein Determination—Protein was determined by the method of Lowry *et al.* (8).

<center>EXPERIMENTAL</center>

<center>*Partial Purification of Enzyme*[11]</center>

Crude Acetylornithinase—For the preparation of acetylornithinase, *E. coli* (ATCC 9637) cells, grown on 3.5 liters of Medium E with 0.5 per cent dextrose, were harvested by centrifugation, suspended in 0.1 M phosphate buffer at pH 7, collected again, and resuspended in phosphate buffer to give a final volume of about 35 ml. The resulting suspension was sub-

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[8] Kindly furnished by Dr. C. Gilvarg.

[9] When necessary, enzyme preparations are diluted in 0.1 M phosphate buffer (pH 7) made 1 mM in glutathione.

[10] The absolute magnitude of the color response was found to vary somewhat from batch to batch of the reagents used.

[11] Carried out at 0–5°.

jected to sonic vibration in a 9 kc. Raytheon oscillator for 30 minutes to yield a crude extract of acetylornithinase.

Protamine Treatment and Precipitation with Ammonium Sulfate—The crude extract obtained was treated with protamine sulfate (210 mg.), dissolved in 0.1 M phosphate at pH 7 (7 ml.). The resulting precipitate was removed by centrifugation. The supernatant solution was treated similarly with half the amount of protamine sulfate previously used, and the precipitate formed was again removed. In the supernatant liquid (37 ml.), ammonium sulfate (16 gm., 60 per cent of saturation) was dissolved with stirring. The resulting precipitate was collected, dissolved in 20 ml. of 0.1 M phosphate at pH 7, and reprecipitated by addition of ammonium sulfate (8.6 gm., 60 per cent of saturation). The precipitate was then collected and dissolved by addition of 0.1 M phosphate buffer at

TABLE I

Color Response Obtained with Ninhydrin Reagent

Sample	Colorimeter reading*	Sample	Colorimeter reading*
Reference blank...............	0	Ornithine, 0.1 μmole..........	160
Substrate "	7	" 0.2 "	318
Enzyme blank...............	2	" 0.3 "	482

* A Klett-Summerson instrument with No. 42 filter was used. The reference blank was set at zero; it gives a reading of about 15 against distilled water set at zero.

pH 7, made 1 mM in glutathione   The final volume of the solution obtained (PS + AS$_{0-60}$) was 17.5 ml.

Fractionation with Ammonium Sulfate—In the solution obtained (PS + AS$_{0-60}$), ammonium sulfate (3.8 gm., 30 per cent of saturation) was dissolved with stirring, and the resulting precipitate was collected and dissolved by addition of phosphate buffer made 1 mM in glutathione (14.5 ml.). The solution obtained (AS$_{0-30}$) had a volume of 15 ml. Solution AS$_{0-30}$ was treated with ammonium sulfate (3.0 gm., 28 per cent of saturation) and the precipitate formed was removed by centrifugation and discarded. The supernatant solution was then treated with ammonium sulfate (0.75 gm., 35 per cent of saturation), and the resulting precipitate was dissolved in phosphate buffer, 1 mM in glutathione, to give a volume of 5 ml. (AS$_{28-35}$).

Fractionation with Acetone—To solution AS$_{28-35}$, acetone (3.5 ml., 41 per cent by volume), previously chilled to 0°, was added with stirring. The precipitate obtained was removed by centrifugation and cold acetone

(4.0 ml., 60 per cent by volume) was added to the supernatant solution. A precipitate formed which was dissolved in phosphate buffer at pH 7 made 1 mM in glutathione to give a volume of 5 ml. The resulting solution was finally dialyzed against the same glutathione-containing buffer. The final dialyzed preparation Ac_{41-60} had a volume of 5 ml. and was stored at $-15°$.

The procedure described results in a 30- to 40-fold purified preparation with an over-all yield of about 25 per cent. The progress of a typical purification is summarized in Table II. Unless otherwise stated, the experiments reported were performed with at least 30-fold purified preparations.

TABLE II

Summary of Enzyme Purification

Fraction*	Total volume	Protein	Enzyme activity	Specific activity	Over-all yield
	ml	*mg. per ml.*	*units per ml.*	*units per mg*	*per cent*
Crude extract	35.0	37.4	950	25	100
PS + AS_{0-60} ...	17.5	21.7	1420	65	75
AS_{0-30}	15.0	9.0	1340	149	60
AS_{25-35} . .	5.0	8.9	2750	309	41
Ac_{41-60} .	5.0	2.1	1680	800	25

* PS, protamine sulfate; AS, ammonium sulfate; Ac, acetone. The subscripts used with AS refer to per cent of saturation; that used with Ac refers to per cent of volume. See the text for details.

Properties of Enzyme and of Reaction

Reaction Products—L-Ornithine has previously been recognized as one of the products of the enzymatic reaction (1). In the present experiments, the formation of 0.93 mole of acetate could be demonstrated per mole of ornithine produced. The acetate was determined by passing the reaction mixture through a bed of the acid form of a sulfonic type ion exchanger, distilling the eluate, and titrating the distillate with alkali. The value obtained is in agreement, within the accuracy of the method used, with the expected equimolar ratio of acetate to ornithine formed.

Extent of Reaction—To ascertain the extent of the reaction, 40 units of enzyme were permitted to act on the substrate. The conditions were standard, except that an incubation period of 3 hours was used. The reaction mixture was then appropriately diluted and assayed for ornithine. The results showed that the reaction had gone to completion within the sensitivity of the method employed.

Time-Course and Dependence on Enzyme Concentration—Under standard

conditions with an amount of enzyme of the order of magnitude of 1 unit, the quantity of substrate cleaved was found to be proportional to time for at least 20 minutes. Over a range of enzyme concentrations, including 0.5 to 3 units per 0.5 ml., the initial rate of cleavage was proportional to enzyme concentration.

Effect of Cobalt—It has been briefly reported that acetylornithinase is stimulated by the cobaltous ion (1). This stimulatory effect is illustrated in Table III. Preincubation of the enzyme in the presence of 0.2 mM cobaltous chloride failed to increase the reaction rate over that obtained under standard conditions. Of a number of other metal ions tested, none proved stimulatory and some were found to be inhibitory (see below).[12]

Effect of Glutathione—The stimulatory effect of added glutathione is also presented in Table III. If enzyme preparations are stored in phos-

TABLE III

Stimulation of Acetylornithinase Activity by Cobaltous Ion and Glutathione

CoCl$_2$	Glutathione	Relative activity*	CoCl$_2$	Glutathione	Relative activity*
mM	mM		mM	mM	
0	1.0	50	0.20	0.2	40
0.02	1.0	70	0.20	1.0	100
0.20	1.0	100	0.20	2.0	100
0.40	1.0	100			

* Each value was obtained with 1 unit of enzyme. The conditions used were standard, except for the variations shown.

phate buffer without glutathione, their activity decreases markedly; such preparations can be largely reactivated by addition of glutathione.[13] A number of reducing agents tested, including thioglycolate, ascorbate, and hydrosulfite, failed to produce the same stimulating and preserving effect as glutathione.

Effect of pH and Buffers—The optimal pH for the action of acetylornithinase was found to be about 7.0, as shown in Table IV. The reaction rate was unchanged when a sodium phosphate buffer at pH 7 was substituted for potassium phosphate. A number of other buffers, in-

[12] Upon addition of alkali in the determination of ornithine, certain heavy metal ions produced extraneous colors which in general faded within several hours; the extraneous color produced by the cobaltous ion was found to fade within 20 minutes.

[13] 30- to 40-fold purified preparations, when stored at −15° in the presence of glutathione, were found to retain their activity for at least several months; however, on repeated thawing and freezing the activity tended to diminish. 12-fold purified preparations, such as AS$_{28-35}$ (see Table II), were more resistant to thawing and freezing.

cluding tris(hydroxymethyl)aminomethane, cacodylate, succinate, bicarbonate, Veronal-acetate, and pyrophosphate, were tested as substitutes for phosphate. The conditions were standard, except for the buffers, and the pH range used was 6.5 to 7.5. None of the buffers examined permitted as high a reaction rate as phosphate at corresponding pH values.

TABLE IV

Effect of pH on Acetylornithinase Activity

pH	Relative activity*
6.0	33
6.5	75
7.0	100
7.5	87
8.0	54

* Each value was obtained with 1 unit of enzyme. The conditions employed were standard, except that the pH was varied by using suitable mixtures of KH_2PO_4 and K_2HPO_4 at constant total phosphate concentration.

TABLE V

Effect of Various Substances on Acetylornithinase Activity

Substance	Relative activity*	Substance	Relative activity*
None	100†	Cu^{++}	60
Mg^{++}	95	Zn^{++}	16
Ca^{++}	95	Ni^{++}	27
Mn^{++}	96	Ethylenediaminetetraacetate	57
Fe^{++}	99	p-Chloromercuribenzoate	43

* Each value was obtained with 2 units of enzyme. p-Chloromercuribenzoate was tested at a concentration of 1 mM under standard conditions. All other substances listed were tested at a concentration of 0.2 mM under standard conditions modified by the omission of cobaltous chloride.

† This value was obtained under standard conditions modified by the omission of cobaltous chloride.

Dependence on Substrate Concentration—The effect of substrate concentration on the reaction velocity was examined over a range of initial concentrations from 1 to 8 mM under otherwise standard conditions. The substrate concentration giving half maximal velocity was calculated (9) to be $K_s = 2.8$ mM.

Metals and Inhibitors—The effect of certain divalent metal ions was studied by substituting them singly for the cobaltous ion.[12] Stimulation of acetylornithinase activity was obtained with the cobaltous ion only.

Salts of magnesium, calcium, manganese, and iron, at the concentrations tested, affected the reaction but slightly; however, salts of copper, zinc, and nickel proved inhibitory (see Table V).

Acetylornithinase was also found to be inhibited by the metal binder, ethylenediaminetetraacetate, and by the sulfhydryl reagent, p-chloromercuribenzoate, as shown in Table V.[14]

Specificity—A number of acylamino acids, including acetyl-DL-alanine, acetyl-DL-valine, acetyl-DL-leucine, acetyl-DL-methionine, acetyl-DL-proline, acetyl-DL-glutamate, chloroacetyl-L-tyrosine, and benzoyl-L-arginine, were tested (6) as possible substrates for acetylornithinase preparations. Under the conditions employed, only acetyl-DL-methionine was found to be deacetylated at a rate approximating that of acetylornithine. The cleavage of acetylmethionine, like that of acetylornithine, was found to be stimulated by the cobaltous ion and by glutathione. The ratio acetylornithinase to acetylmethioninase activity was found to be approximately constant in crude, 12-fold, and 30-fold purified preparations. Extracts of the ornithine-requiring mutant 160-37 of *E. coli*, which had previously been shown to contain no detectable acetylornithinase (5, 1), have now been shown to be devoid also of detectable acetylmethioninase activity. It therefore appears that the cleavage of acetylmethionine is largely, if not exclusively, mediated by acetylornithinase.

DISCUSSION

The present study has revealed that acetylornithinase resembles other acylases (10, 11) and certain peptidases (12) in a number of features. These features include the observed stimulation by the cobaltous ion, which in the present case appears to be specific. The stimulating and preserving action of glutathione and the inhibiting action of p-chloromercuribenzoate are also shared with functionally related enzymes. For example, prolidase (13) from animal (14) and microbial (15) sources is similar to acetylornithinase in being both a "metal" and "sulfhydryl" enzyme. Furthermore, the enhanced reaction rates with phosphate compared to those obtained with other buffers are not without precedent. In some cases, this enhancement has been ascribed to the selective binding of toxic metal ions by the phosphate (*cf.* (12)).

In view of the voluminous earlier work on acetylamino acids and enzymes that participate in their metabolism, it seems of special interest that acetylornithinase was found to have a definite biosynthetic function, namely the catalysis of a step in the biosynthesis of ornithine (1). More-

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[14] Although glutathione was found to antagonize the inhibitory effect of p-chloromercuribenzoate, the latter was tested under standard conditions (in the presence of glutathione) because of the instability of the enzyme in the absence of glutathione.

over, the function of this acylase appears to be an essential one, at least under the conditions employed, since it could be shown (5, 1) by means of an *E. coli* mutant that absence of detectable acetylornithinase is associated with a growth requirement for ornithine (or an equivalent metabolite).

Acetylornithinase activity has been demonstrated in all of several Enterobacteriaceae tested, including strains K-12 and B of *E. coli* and strains of *Aerobacter*, *Klebsiella*, *Erwinia*, *Serratia*, *Proteus*, *Salmonella*, and *Shigella* (16).[15]   In contrast, no appreciable acetylornithinase activity could be detected in *Neurospora crassa* (17), several other fungi, or certain Bacillaceae (16).[15]   The presence of acetylornithinase activity in the Enterobacteriaceae suggests that they, like *E. coli*, produce ornithine via acetylated intermediates.   On the other hand, the absence of detectable acetylornithinase activity in the Bacillaceae and fungi provides evidence that these organisms do not use an acetylation mechanism in ornithine synthesis.   It is not unlikely that the Bacillaceae as well as the fungi synthesize ornithine from glutamic γ-semialdehyde, as has been reported for *N. crassa* (17) and *Torulopsis utilis* (18).

SUMMARY

Acetylornithinase has been extracted from *Escherichia coli* and partially purified by a procedure including treatment with protamine and fractionation with ammonium sulfate and acetone.

Acetylornithinase appears to be specifically stimulated by the cobaltous ion and by glutathione; it is inhibited by the divalent ions of copper, zinc, and nickel as well as by ethylenediaminetetraacetate and *p*-chloromercuribenzoate.

Under the conditions used, the substrate concentration giving half maximal velocity is 2.8 mM, and the pH optimum of the enzymatic reaction is about 7.0.

Acetylornithinase appears capable of deacetylating N-acetylmethionine at a rate approximating that obtained with N^α-acetylornithine.

Enzyme assays have been carried out by means of a convenient modification of the ninhydrin method of determining ornithine.

A simple growth medium for the organisms used has also been described.

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THE EFFECT OF FAT INTAKE ON INCORPORATION OF ACETATE-2-C^{14} INTO LIVER LIPIDE AND EXPIRED CARBON DIOXIDE

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WITH THE TECHNICAL ASSISTANCE OF MARCIA M. STONE

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Most of the studies of the effect of diet on utilization of tagged acetate have been carried on with liver slices or similar preparations, and very few with intact animals. In the *in vitro* experiments by Tomkins, Sheppard, and Chaikoff (1), the C^{14}O$_2$ recoveries from added acetate-1-C^{14} were highest for the liver slices from rats fed a 47 per cent fat diet for 8 days, intermediate for those given 10 per cent fat, and least for rats fed no fat. However, Lyon, Masri, and Chaikoff (2) showed that, for fasted rats killed 6 hours after a single intubation of 2.5 ml. of corn oil, the C^{14}O$_2$ recoveries from doubly labeled acetate added to liver slices were depressed. Recoveries reached normal levels in rats killed 12 to 18 hours after the fat was given.

Alfin-Slater *et al.* (3) found that hepatic synthesis of cholesterol in intact rats fed deuterium was the same on low and high fat diets. Most similar experiments reported in the literature do not take into consideration the change in calorie and protein intake or utilization which is likely to result from alteration of the fat content of the diet. Data from studies of fat synthesis by liver slices are difficult to interpret in terms of the metabolism of the whole animal.

The present paper reports measurements of C^{14}O$_2$ excretion and of C^{14} in liver fat and cholesterol made at frequent intervals following the intraperitoneal injection of a tracer dose of acetate-2-C^{14} into normal, non-fasted male rats.

EXPERIMENTAL

Dietary Treatment of Animals—Diets[1] were made to contain 5 or 40 per

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* The data are taken from a thesis submitted by Esther Goossen Brice in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Nutrition, University of California, June, 1954.

[1] The composition of the diets, in per cent, was as follows: Diet a, "low fat," casein 18, fat (Primex) 5, salts (Hubbell) 2.5 (4), sucrose 73.5, fat-soluble vitamin mix 1; calories, per 100 gm., 420; Diet b, "high fat," casein 25.3, fat 40, salts 3.75,

cent fat and to furnish approximately the same amounts of protein and salt per 100 calories. Data are reported for ten series, each of three litter mate male rats which had eaten food of equivalent energy and protein content for 3 weeks following weaning. A series consisted of one rat given the 5 per cent fat diet *ad libitum* (Diet a), one rat pair-fed with isocaloric amounts of the 40 per cent fat diet (Diet b), and one given the 40 per cent diet *ad libitum* (Diet c). The last group was necessary because the larger bulk of the low fat diet tended to decrease food consumption below that normal for the diet high in fat.

Methods—After 3 weeks on diet, each rat was injected intraperitoneally with acetate-2-C^{14} in tracer amounts (2.7×10^6 to 3.6×10^6 c.p.m.). The animal was placed immediately in a metabolism chamber where it had access to food and water. Respiratory CO_2 was trapped in CO_2-free NaOH, and studied at frequent intervals (Fig. 1) after injection of the acetate. Aliquots of the labeled carbonate were precipitated as $BaCO_3$, and total expired CO_2 was gravimetrically determined. For counting, $BaCO_3$ samples were plated in duplicate on 2 inch aluminum disks. Radioactivity was determined with a Geiger-Müller tube with a thin mica window, and proper corrections were made for background and self-absorption and for dilution of radioactive $BaCO_3$ by addition of non-isotopic Na_2CO_3 when necessary. At the end of the last CO_2 collection period, the rat was killed by decapitation, and the liver was removed, weighed, and divided into weighed aliquots. Within 6 to 8 minutes, either saponification of the liver lipides was under way or the sample was in the deep freeze, stored for later analysis.

For saponification, the liver samples were placed in hot alcoholic KOH and refluxed gently on a steam bath overnight. Most of the alcohol was removed by evaporation, the extract was diluted with water, and the non-saponifiable fraction was extracted with five portions of petroleum ether, each having at least 5 times the volume of the hydrolysate. Further extraction removed no measurable radioactivity.

The petroleum ether extracts were combined, and the volume was reduced by evaporation. The combined extracts were transferred quantitatively to a separatory funnel and washed repeatedly with 15 to 20 ml. portions of water to remove the soaps. After the washings were no longer alkaline to phenolphthalein, the petroleum ether extracts were dried over

sucrose 29.45, vitamin oil mix 1.5, and calories, per 100 gm., 592. B complex vitamins were given separately in 20 per cent alcohol. The daily dose levels for vitamins, in micrograms, were thiamine 20, riboflavin 40, pyridoxine 20, pantothenic acid 100, niacin 66, *p*-aminobenzoic acid 100, folic acid 20, biotin 2, and inositol 2.5 mg., choline 5 mg., vitamin A, 100 i.u., vitamin D 10 i.u., mixed tocopherols 1 mg., menadione 49 γ. For purposes of pair feeding, 71 gm. of Diet b were considered equivalent to 100 gm. of Diet a.

Na₂SO₄ and made to volume. Aliquots were used for counting and for determination of total cholesterol by the method of Sperry and Webb (5).

The saponified aqueous mixture was combined with the water washings from the petroleum ether extract and acidified to bromocresol green with HCl. The fatty acids were extracted with five portions of petroleum ether, which were combined, and the volume was reduced by evaporation. After being washed three to five times with water, the extract was made to volume. Aliquots were used for counting and for determination of total fatty acids by the method of Bloor (6).

For determination of radioactivity, the cholesterol was precipitated as digitonide, carefully purified, and plated from an acetone suspension. After the counting operation, the amount of digitonide on each plate was analyzed by the method of Sperry and Webb. Counts were corrected to zero mass and expressed as noted below. Radioactivity of the fatty acids was measured by direct plating of 1 ml. aliquots of the petroleum ether extract. Counts were made and computed as for infinitely thin samples. *Specific activities* were calculated as counts per minute per mg. of fatty acid on the basis of analyses of the petroleum ether extracts. *Percentage incorporation* was computed as mg. per liver times the specific activity divided by the counts of acetate-2-C^{14} given.

Results

$C^{14}O_2$ *Excretion*—Typical curves for the relative specific activities of the expired CO_2 are given in Fig. 1. The maximal specific activity for each rat was reached within $\frac{1}{2}$ to 1 hour after the injection of the labeled acetate at both levels of ingested fat. In each litter group, the specific activities at this time were higher for the rats fed the 40 per cent fat diet than for those fed the 5 per cent fat diet. After 1 to 2 hours, the specific activity curves for the rats fed the high fat diet closely paralleled those for the rats fed the low fat diet.

The time-course of the $C^{14}O_2$ excretion is shown in Fig. 2. The data indicate, as do the specific activities, that, in all of a considerable series of rats, incorporation of a large portion of the injected C^{14} into expired CO_2 took place during the first two half-hour intervals after injection of the acetate-2-C^{14}. After the 3rd hour, the rate of excretion was markedly lower and remained more or less constant up to 24 hours. This suggests that the later excretion of $C^{14}O_2$ represents oxidation, not of acetate but of other substances into which the acetate-C^{14} had been incorporated. The indicated rapidity of the processes involved in acetate absorption, distribution, metabolism, and excretion as CO_2 is striking.

The total output of CO_2 was not notably affected by the level of fat fed nor by the slightly higher calorie intake of the *ad libitum* group. Mean
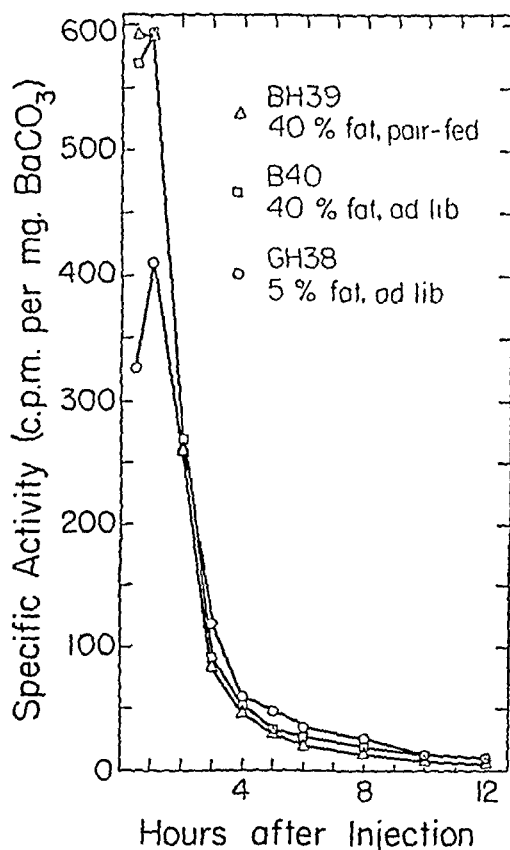
FIG. 1. Typical curves for specific activity of expired CO_2 in a series of litter mate rats injected with acetate-2-C^{14}. BH39, B40, and GH38 indicate the ear-marks and the color of the rats.
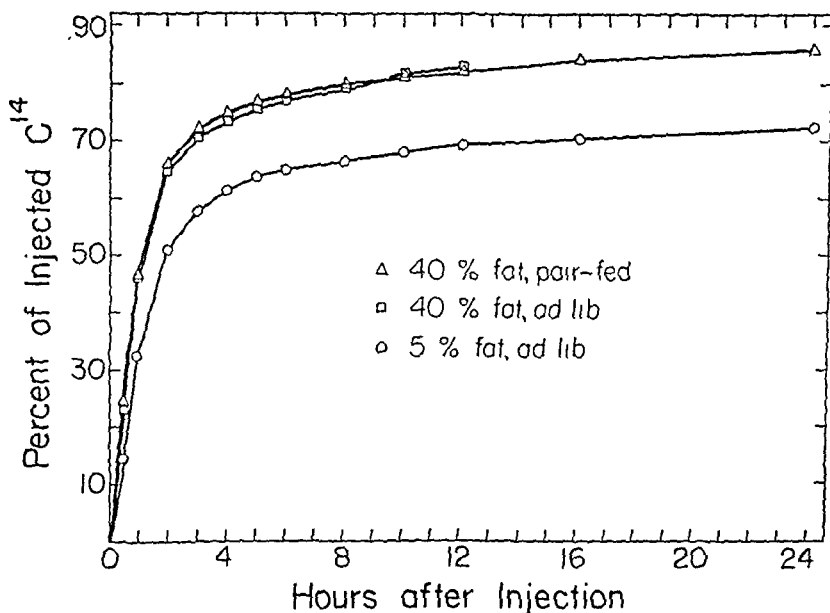

FIG. 2. Mean cumulative excretions of $C^{14}O_2$ following intraperitoneal injection of acetate-2-C^{14} for ten series of three litter mate rats each.

values, expressed as mg. of $BaCO_3$ per hour, were 2579 for the rats fed 5 per cent fat, 2444 for the pair-fed group with 40 per cent fat, and 2500 for

TABLE I

Liver Lipides Following Injection of Acetate-2-C^{14}

Litter group No.	Diet No.*	Time after injection, hrs.	Liver fatty acid			Liver cholesterol			Per cent C^{14} expired as $C^{14}O_2$‡
			Per cent moist weight	Specific activity	Per cent incorporation, C^{14}	Per cent moist weight†	Specific activity	Per cent incorporation, C^{14}†	
I	a	0.5	3.31	1104	5.88	0.19	394	0.12	10.2
	b	0.5	9.16	37	0.52	0.49	322	0.21	16.3
II	a	0.5	4.01	88	0.57	0.20	104	0.03	15.2
	b	0.5	7.50	49	0.56	0.26	345	0.13	27.5
	c	0.5	6.92	60	0.80	0.23	214	0.09	21.6
III	a	1.0	2.87	510	2.99	0.18	473	0.17	27.7
	b	1.0				0.25	451	0.18	45.7
	c	1.0	9.83	33	0.57	0.21	122	0.05	45.6
IV	a	3.0	3.81	1059	7.21	0.19	848	0.29	53.0
	b	3.0	6.45	17	0.18	0.30	426	0.10	73.3
	b	3.0	4.98	23	0.17	0.22	261	0.09	71.5
V	a	3.0	2.94	463	2.87	0.18	98	0.04	55.7
	b	3.0	6.88	10	0.11	0.35	99	0.06	74.9
	c	3.0	8.14	20	0.30	0.35	131	0.09	74.8
VI	a	5.0	3.60	222	1.35	0.17	196	0.06	70.3
	b	5.0	7.98	29	0.43	0.33	220	0.14	78.2
	c	5.0	8.95	19	0.33	0.36	62	0.04	80.4
VII	a	8.0	3.63	388	2.74	0.22	291	0.13	65.1
	b	8.0	6.69	22	0.23	0.18	155	0.04	80.1
	c	8.0	12.21	68	1.62	0.23	476	0.21	75.1
VIII	a	12.0	3.44	150	1.33	0.15	167	0.07	67.3
	b	12.0	6.92	17	0.25	0.12	116	0.03	79.0
IX	a	12.0	3.93	176	1.25	0.11	383	0.07	72.5
	b	12.0	6.25	24	0.35	0.17	274	0.10	82.6
	c	12.0	7.41	31	0.39	0.17	146	0.05	80.5
X	a	24.0	3.41	60	0.41	0.20	114	0.05	79.9
	b	24.0	6.37	11	0.14	0.17	153	0.05	88.3

* Diet a, 5 per cent fat *ad libitum;* Diet b, 40 per cent fat pair-fed; Diet c, 40 per cent fat *ad libitum.*

† Based on extraction from alkaline suspension, and determination by the Sperry-Webb procedure.

‡ Cumulative.

the rats given 40 per cent fat *ad libitum.* This is important because it indicates that the difference in percentage incorporation of the acetate methyl carbon into CO_2 was not due to any effect of the diet on the amount of CO_2 produced, or, if the CO_2 output is used as a criterion, to any increase

in the metabolic rate of the rats caused by the ingestion of a diet high in fat.

Fatty Acids—The livers of both groups of rats fed 40 per cent fat were significantly richer in fat than were those of their litter mates fed 5 per cent fat. The mean for the rats given the 40 per cent diet *ad libitum* was 8.9 ± 1.9 per cent,[2] for the pair-fed groups 6.9 ± 1.1 per cent, and for the 5 per cent groups 3.5 ± 0.4 per cent. Conversely, the specific activity of the liver fatty acids was much greater in the rats fed 5 per cent fat than in either group fed 40 per cent fat, as was the percentage incorporation of the injected C^{14} into the liver fatty acid (Table I).

In general, the data indicate that a high fat diet lowers the amount of liver fatty acid synthesized from acetate, but does not stop the process.

Cholesterol—The data for cholesterol were inconclusive. They varied widely and demonstrated no consistent relationship either to fat intake or to time after injection (Table I).

DISCUSSION

$C^{14}O_2$ *Excretion*—The data indicate that the level of dietary fat influenced the rate of oxidation of the tagged acetate only while the acetate, as such, was being actively oxidized. There is little or no evidence that diet influenced oxidation of the other substances into which the tagged acetate was incorporated. This concept is in agreement with the findings of Harper *et al.* (7), who studied serum acetate, exhaled CO_2, and urinary excretion of acetate in a dog after injection of tagged acetate; they reported that the injected acetate was completely metabolized during the rising phase of the specific activity curve for CO_2 and disappeared from the serum at the time the specific activity of exhaled CO_2 reached a maximum.

The concept of a rapid initial oxidation of acetate *per se*, followed by a slow and steady oxidation of secondary compounds derived from it, seems reasonable in view of what is known of the oxidation of fatty acids and cholesterol. For instance, when palmitic acid labeled with C^{14} at its 6th carbon was injected (as its triglyceride), Lerner *et al.* (8) found that fasted rats excreted only 36 to 59 per cent of the C^{14} as CO_2 in 24 hours. Similarly, only 31 per cent of the C^{14} from cholesterol labeled at position 26 was excreted as $C^{14}O_2$ in 24 hours (9).

After 12 hours, only 67.3 and 72.5 per cent of the C^{14} given in the present study had been recovered as $C^{14}O_2$ in the rats fed 5 per cent fat, whereas 79.0 and 82.6 per cent of the C^{14} dose had been expired as $C^{14}O_2$ by the pair-fed rats given 40 per cent fat. Similar trends were noted at other time intervals. The obvious inference is that the animals on the low fat diet incorporated a larger percentage of the ingested acetate into more

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[2] Standard deviation.

slowly metabolized compounds. It seems probable that, in the animals fed the diet high in fat, the endogenous acetate "pool" may be larger than that in the rats fed less fat, because of acetate formation in β oxidation. Wick and Drury (10) have reported that the rate of oxidation of injected acetate-2-C^{14} by extrahepatic tissues of non-fasted rabbits is proportional to the concentration of acetate in the animal.

The formation of $C^{14}O_2$ from injected acetate was not affected by the slight restriction in food intake of the pair-fed rats. Because data on the effect of fasting on rate of oxidation of acetate were not in good agreement (11, 12), rats were allowed access to food before and after injection of the acetate. Differences in food eaten by individual animals during this period may have increased variability in the data.

The prompt appearance of the injected C^{14} in liver fat and cholesterol supports the conclusions of Stetten and Schoenheimer (13) and of Van Bruggen et al. (14) that the synthesis of these lipides is normally rapid. Our finding that the liver lipides of the rats with fatty livers were taking up C^{14} shows that these livers were synthesizing fat. Since the rate of oxidation of the C^{14}-labeled acetate was greater and the rate of synthesis of labeled fatty acid was less in the rats fed the diets high in fat, the data indicate that there is some homeostatic control of fatty acid synthesis.

Several studies with liver slices (15) indicate that the rate of formation of liver fat decreases when the fat content of the liver is increased. The lowering of the specific activity of the liver fatty acids which occurred after the 3rd hour following C^{14} injection was marked in the rats on the low fat diet, but was much less in those fed 40 per cent fat.

SUMMARY

The effect of varying the fat intake without altering the intake of calories and protein was studied in relation to excretion of $C^{14}O_2$ and synthesis of labeled liver lipides following a single intraperitoneal injection of acetate-2-C^{14}. Weanling male rats, litter mates in groups of three, were fed for 3 weeks previous to injection either (a) a diet containing 5 per cent fat and 18 per cent casein ad libitum, (b) a diet containing 40 per cent fat in amounts adjusted to match the calorie and protein intake of the first group, or (c) the 40 per cent fat diet ad libitum.

For about 1 hour after injection, the specific activity of expired CO_2 was significantly higher with Diets b and c than with Diet a. Both specific activity and percentage incorporation of injected C^{14} into exhaled CO_2 fell markedly in all rats about 1 hour after injection and remained low and fairly constant from about 2 to 24 hours. The total CO_2 output was not altered by the percentage of fat in the diet. Cumulative total expiration of $C^{14}O_2$ during a 12 hour period averages 69.9 per cent of the dose for the

rats fed Diet a and 80.8 per cent for the pair-fed rats on Diet b. The data indicate that a high fat diet leads to an increased rate of oxidation of acetate, possibly concomitant with accelerated acetate production by β oxidation of fatty acids.

Fat content of the livers averaged 3.5, 6.9, and 8.9 per cent, respectively, for Diet groups a, b, and c. The label appeared promptly in the liver fat and cholesterol of rats fed both low and high fat diets. Specific activity and percentage of incorporation of the injected C^{14} into fatty acid were higher in the livers of the rats fed 5 per cent fat than in those given the high fat diet. The fall in specific activity with time was marked after 2 to 5 hours in the low fat group, and less so in the high fat groups.

The variability of the activity of the liver cholesterol was so great that it permits no conclusions.

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OBSERVATIONS ON THE STABILITY OF GROWTH HORMONE*

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A method for the preparation of highly purified anterior pituitary growth hormone, based on the methods of Li and Pedersen (1) and of Wilhelmi *et al.* (2) has recently been described (3). Growth hormone thus prepared was found to differ significantly in stability from that reported by Li and Papkoff (4). By means of electrophoretic analysis the latter investigators observed the formation of a second component in previously homogeneous growth hormone solutions exposed to pH 4 or less, and to pH 8.4 or 9.4 or above, at 25°. The half time of formation of the new component was estimated to be 32 hours at pH 9.4. Growth hormone prepared by the method of Ellis *et al.* (3) consistently showed no electrophoretic change under the mild conditions of pH 8.6 or 9.6. This discrepancy necessitated a reexamination of the stability of growth hormone over a wide range of pH, particularly between pH 4 and 8.6, which has not been hitherto reported. In order to determine whether there were any further dissimilarities, it was important to reexamine the effect of some of the conditions used by Li and Papkoff (4). The results obtained show that growth hormone is biologically and electrophoretically stable between pH 5 and 11.5 at 25°. At greater extremes of pH the lability of growth hormone was comparable to that observed by Li and Papkoff.

EXPERIMENTAL

The preparations used in this study have been characterized in terms of electrophoretic behavior, solubility properties, and biological potency. Purified growth hormone obtained by the procedure previously described (3) was further fractionated on the basis of solubility in distilled water. The solubility of the starting material in distilled water with respect to pH is shown in Fig. 1. The point of minimal solubility was at about pH 8.0. From a 0.5 per cent protein solution at pH 4.0, the precipitates which formed on successive adjustments to pH 5.9 and 6.9 were removed by centrifugation. The precipitate formed at pH 8.0 was used in the studies described below. The electrophoretic patterns of the precipitates at pH

6.9 and pH 8 are presented in Fig. 2. Inspection of these patterns shows that the latter preparation was more nearly homogeneous.

All electrophoretic determinations were performed at 0.5° with a 2 cc. cell (open type) Perkin-Elmer electrophoresis apparatus. The mobilities were calculated from the descending limb from the conductivity of the pure buffer solution.
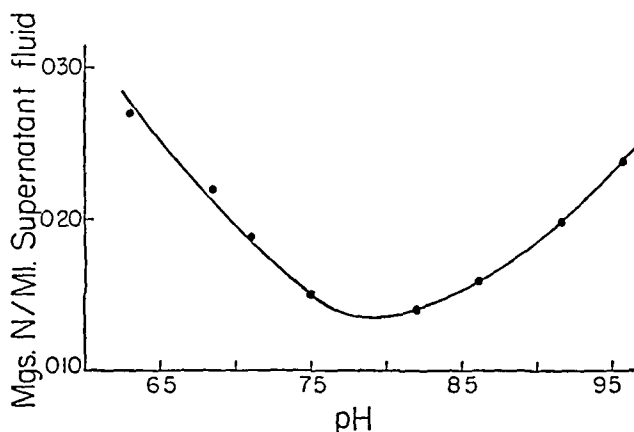

FIG. 1. The solubility of growth hormone in distilled water as a function of pH. An approximately 0.2 per cent solution of purified growth hormone was equilibrated for 15 minutes at the indicated pH. An aliquot of the suspension was centrifuged and the clear supernatant solution analyzed for nitrogen.
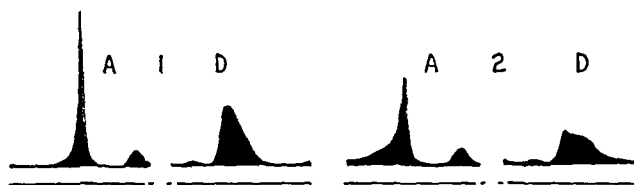

FIG. 2. Ascending (A) and descending (D) electrophoretic patterns of the protein precipitated at pH 8.0 and pH 6.9 (Patterns 1 and 2, respectively), after electrophoresis for 45 minutes in 0.015 M NaOH-0.03 M glycine buffer, pH 9.6, potential gradient 19.0 volts per cm.

Growth hormone activity was estimated by the 4 day tibia test (increase in width[1] of the uncalcified portion of the proximal epiphyseal cartilage of the tibia compared with that of the untreated hypophysectomized rat) (5). The growth activity retained by treated preparations was expressed as per cent of the increase given by the untreated growth hormone. Untreated growth hormone was always assayed simultaneously with the treated preparations. This was found to be essential, since the responsiveness of the hypophysectomized rats to a given preparation of growth hormone fluctuates from week to week.

[1] Δ width in Tables I and II.

Results

Effect of pH 2.5 to 4—From the data of Table I, it is evident that growth hormone consistently decreased in activity on standing in acid solutions at

TABLE I

Biological Activity and Electrophoretic Composition of Growth Hormone Following Treatment in Acid Solutions

Treatment of growth hormone		Dose	No. of rats	Tibia test			Electrophoresis at pH 4.0;‡ slow component
Solvent	Time			Width*	Δ width†	Activity retained	
	hrs.	*γ*		*μ*	*μ*	*per cent*	*per cent*
pH 2.5, glycine buffer	3	50	7	221 ± 5	62	68	34
" 2.5, " "	12	50	8	201 ± 7	42	46	24
Untreated hormone		50	8	250 ± 10	91	100	
Uninjected control		0	6	159 ± 7			
pH 2.5, glycine buffer	40	50	15	180 ± 4	24	34	0
Untreated hormone		50	15	226 ± 6	70	100	
Uninjected control		0	10	156 ± 3			
1 N HC₂H₃O₂	6	50	10	210 ± 6	43	71	49
0.1 N HC₂H₃O₂	12	50	10	214 ± 6	47	77	68
Untreated hormone		50	10	228 ± 5	61	100	
Uninjected control		0	6	167 ± 9			
1 N HC₂H₃O₂	12	50	10	226 ± 5	66	85	31
0.1 N HC₂H₃O₂	24	50	10	207 ± 12	47	60	35
Untreated hormone		50	8	238 ± 7	78	100	
Uninjected control		0	8	160 ± 4			
2 N HC₂H₃O₂	12	50	8	232 ± 7	73	80	
Untreated hormone		50	9	250 ± 10	91	100	
Uninjected control		0	6	159 ± 7			
pH 4.0, acetate buffer	24	80	12	251 ± 7	83	100	54
" 4.0, " "		40	12	221 ± 7	53	100	
Untreated hormone		80	9	253 ± 5	85	100	
" "		40	9	218 ± 6	50	100	
Uninjected control		0	8	168 ± 7			
pH 4.0, acetate buffer	40	50	15	189 ± 5	33	47	10
Untreated hormone		50	15	226 ± 6	70	100	
Uninjected control		0	10	156 ± 3			

* Mean width of uncalcified portion of the proximal epiphyseal cartilage ± the standard error.

† Difference from the mean control width.

‡ 0.03 M NaOAc-0.145 M HOAc, pH 4.0, ionic strength 0.03.

25°. The greatest loss occurred in buffer (0.1 N HCl-0.2 N glycine) at pH 2.5, in which 54 per cent of the activity was lost after 12 hours of treatment. In acetic acid solutions of the corresponding pH, obtained by dissolving the hormone in 1 or 2 N acetic acid, the loss of activity was not so rapid, only 15 to 20 per cent being lost after 12 hours. There was no discernible difference in the extent of inactivation when the preparations were treated in 2 N, 1 N, or 0.1 N acetic acid solutions for 12 hours. The treatment of growth hormone in 0.03 M sodium acetate-0.145 M acetic acid buffer at pH 4.0 for 24 hours resulted in no detectable loss of activity; however, after 40 hours one-half of the activity was lost.

Electrophoretic examination of these treated samples of growth hormone showed the presence of two components, the fast moving component having been absent in the untreated starting material. The amount of the fast component, as determined by measurement of the area under this peak, was a function of the time of exposure to the acid solutions. The slow component was progressively converted to the fast component with increasing time of exposure to the acid solutions. The time for 50 per cent conversion was $1\frac{1}{2}$ hours in glycine buffer at pH 2.5, 6 hours in 1 N acetic acid, 18 hours in 0.1 N acetic acid, and 24 hours in acetate buffer of pH 4.0. It is noteworthy that no loss of biological activity was detected in growth hormone which had been treated for 24 hours in acetate buffer at pH 4.0, although 46 per cent conversion into the fast component had occurred (Fig. 3). Table I illustrates the degree of conversion to the fast moving component and the accompanying per cent of biological inactivation under the various conditions employed.

The electrophoretic mobility of untreated growth hormone was 6.4×10^{-5} sq. cm. per second per volt in 0.03 M sodium acetate-0.145 M acetic acid buffer at pH 4.0, 0.03 ionic strength. The fast moving component resulting from the acid treatments had a mobility of 7.4×10^{-5} sq. cm. per second per volt. The mobility of the slow moving component was 5.5×10^{-5} sq. cm. per second per volt.

Effect of pH 5.0 to 11.5—The electrophoretic patterns and biological potency of growth hormone were studied after treatment for 12 and 24 hours in the range of buffers[2] from pH 5.0 to 11.5 at 25°. Under these circumstances, no loss of biological potency was detectable. Electrophoretic patterns of the treated hormone were obtained both in 0.03 M NaOAc-0.145 M HOAc buffer at pH 4.0 and in 0.03 M NaOH-0.077 M glycine buffer at pH 9.6, 0.03 ionic strength. No modification in electrophoretic patterns

[2] The pH and the buffer composition of these solutions were as follows: pH 5.0, 0.1 M NaOAc-0.048 M HOAc; pH 5.5, 0.1 M NaOAc-0.016 M HOAc; pH 7.8, 0.004 M NaH$_2$PO$_4$-0.032 M Na$_2$HPO$_4$; pH 8.6, 0.026 M Na$_2$B$_4$O$_7$-0.022 M HCl; pH 9.5, 0.04 M Na$_2$B$_4$O$_7$-0.02 M NaOH; pH 9.6, 0.05 M NaOH-0.13 M glycine.

from that of the untreated hormone could be found.   A change in electrophoretic pattern was observed under one set of cricumstances only, 0.05 M Na_2HPO_4-0.034 M NaOH buffer at pH 11.5 and 25° for 27 hours.   Under these conditions 25 per cent conversion to the fast component was observed; the biological activity was not decreased.

Li and Papkoff (4) had found that a second component appeared after treatment at pII 8.4 or 9.4.   Since the absence of such an effect under the conditions reported here might result from differences in methods of hormone preparations, growth hormone was prepared as closely as possible by
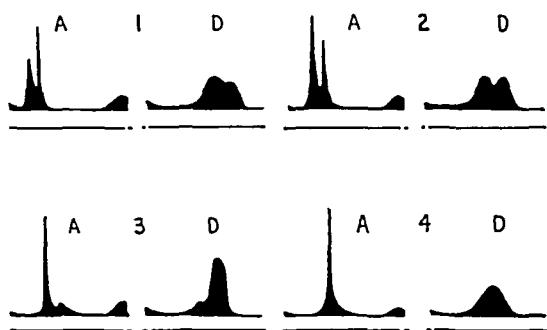

Fig. 3. Ascending (A) and descending (D) electrophoretic patterns showing the progressive increase of the fast moving component.   Growth hormone in Patterns 1, 2, and 3 after treatment for $12\frac{1}{2}$, 24, and 40 hours with 0.03 M NaOAc-0.145 M HOAc buffer at pH 4.0.   Pattern 4 shows growth hormone after treatment with 0.05 M NaOH-0.13 M glycine buffer at pH 9.6 for 24 hours.   This pattern is indistinguishable from that of untreated growth hormone and shows the absence of an effect of treatment at pH 9.6 on growth hormone.   Patterns after 45 minutes electrophoresis in 0.03 M NaOAc-0.145 M HOAc buffer, pH 4.0, at a potential gradient of 13.6 volts per cm.

the method of Wilhelmi et al. (2) and that of Li and Pedersen (1).   These preparations also showed no modification of electrophoretic pattern after exposure to glycine or borate buffers at pH 9.6 for 24 hours.

Effect of 0.1 N NaOH—In 0.1 N NaOH the complete growth activity was retained after up to 3 hours of treatment at 25°; after 6 hours 67 per cent of the activity was retained (Table II).   The protein content of the hormone solutions so treated was so high (20 mg. per cc.) that dilution of an aliquot to the injection level (0.025 mg. per cc.) was sufficient to bring the pH to between 8.5 and 9.5.   The protein was therefore at no time exposed to acid pH before injection.   When the alkali-treated protein solutions were adjusted to pH 4.0 with dilute acid, evolution of H_2S resulted.   Biological assay of the solutions obtained after adjustment to pH 4.0 showed a loss of activity.   Preparations treated with alkali for 3 hours, then ad-

justed to pH 4.0, retained 38 to 56 per cent of the original activity, whereas these preparations retained 100 per cent of their activity if the pH did not fall below pH 8.5 to 9.5.

TABLE II

Biological Activity and Electrophoretic Composition of Growth Hormone Following Treatment in Alkaline Solutions

Treatment of growth hormone		Dose	No. of rats	Tibia test			Electrophoresis at pH 4.0;‡ slow component
Solvent	Time			Width*	Δ width†	Activity retained	
	hrs.	*γ*		*μ*	*μ*	*per cent*	*per cent*
pH 11.5, phosphate buffer	12	50	10	250 ± 5	80	100	100
" 11.5, " "	27	50	9	273 ± 6	103	120	75
Untreated hormone		50	10	251 ± 9	81	100	
Uninjected control	*0*		*8*	*170 ± 11*			
0.1 N NaOH	¼	50	10	238 ± 8	63	100	
0.1 " "	½	50	10	235 ± 6	60	100	61
0.1 " "	1	50	9	246 ± 14	71	100	40
0.1 " "	2	50	10	246 ± 5	71	100	
0.1 " "	3	50	10	237 ± 8	62	100	14
Untreated hormone		50	10	241 ± 9	66	100	
Uninjected control		0	8	175 ± 6			
0.1 N NaOH§	3	50	10	210 ± 8	23	38	
Untreated hormone		50	10	248 ± 8	61	100	
Uninjected control		0	8	187 ± 4			
0.1 N NaOH	6	50	9	218 ± 10	58	67	0
0.1 " " §	3	50	10	209 ± 13	49	56	
Untreated hormone		50	10	247 ± 11	87	100	
Uninjected control		0	8	160 ± 6			

* Mean width of uncalcified portion of the proximal epiphyseal cartilage ± the standard error.

† Difference from the mean control width.

‡ 0.03 M NaOAc-0.145 M HOAc, pH 4.0, ionic strength 0.03.

§ Adjusted to pH 4.0 after treatment with 0.1 N NaOH.

After treatment of growth hormone for ½, 1, 2, or 3 hours in 0.1 N sodium hydroxide, electrophoretic studies were conducted in 0.03 M NaOH-0.077 M glycine buffer at pH 9.6 and 0.03 M NaOAc-0.145 M HOAc buffers at pH 4.0, 0.03 ionic strength. In both buffers a fast moving peak appeared, which progressively increased in area as a function of time of exposure to alkali. The half time of conversion to the fast component was 45 minutes as calculated from electrophoretic patterns obtained at

pH 4.0, and 60 minutes as calculated from patterns at pH 9.6. The mobilities of the fast and of the slow components in 0.03 M sodium acetate-acetic acid buffers at pH 4.0 were, respectively, 7.1×10^{-5} and 5.2×10^{-5} sq. cm. per volt per second, while in 0.03 M sodium hydroxide-glycine buffer at pH 9.6 the mobilities of the fast and slow components were -4.4 and -2.1×10^{-5} sq. cm. per volt per second. The electrophoretic patterns obtained were similar to those shown in Fig. 3. The growth hormone before treatment had a mobility of -2.3×10^{-5} sq. cm. per volt per second in the latter buffer.

Although all growth hormone preparations after treatment in 0.1 N NaOH showed the presence of a fast moving component when the electrophoresis was conducted in buffer at pH 9.6, this was not consistently characteristic on electrophoresis in buffer at pH 4.0. In one growth hormone preparation out of six (all prepared by the same method (3) and treated with 0.1 N NaOH) there was a scarcely perceptible shoulder on the single peak instead of two peaks after electrophoresis at pH 4.0. Since this preparation did not differ significantly in electrophoretic homogeneity or in biological potency, it is difficult to explain the absence of the distinct fast moving peak.

DISCUSSION

From the data presented, it appears that growth hormone under the conditions described has a range of maximal stability from pH 5.0 to 11.5. In this range no change in electrophoretic pattern or detectable biological inactivation occurred up to 24 hours at 25°. In contrast to these results, Li and Papkoff have observed that growth hormone is gradually converted to the fast moving electrophoretic form at pH 9.4 at a rate of approximately 50 per cent per 32 hours, without loss of biological activity. It has not been possible to reproduce their electrophoretic results, though growth hormone prepared by three different methods has been studied. At the present time it is not possible to present a reason for the conflicting observations. The use of unequivocally homogeneous preparations of growth hormone would be desirable, since it is conceivable that slight impurities would affect either the denaturation or the resolution during electrophoresis. This criticism applies also to the work of Li and Papkoff.

It is of interest to compare the electrophoretic analyses reported here for growth hormone treated in more concentrated alkali (0.1 N NaOH) with those in buffer at pH 9.4 observed by Li and Papkoff. The percentage of the fast moving component as determined in both buffers, pH 4.0 and 9.6, was found to increase with time of alkaline treatment, from which it was inferred that the fast moving components observed in the two buffers represented the same entity. If this is true, the curves of the

mobilities plotted against pH of the fast and slow components would then intersect at some point. In the Li and Papkoff studies, the curves of the mobilities plotted against the pH, obtained from electrophoretic analysis of growth hormone treated at pH 9.4, did not intersect. Hence, the faster moving component which appeared on electrophoresis in buffer at pH 4.0 could be identified as the slower moving component present in alkaline buffers. Although in the present study the formation of two electrophoretic components was not observed after treatment with alkaline buffers of pH 8.6 and 9.6, two peaks did appear on electrophoresis after treatment with 0.1 N NaOH. It is significant that in the latter instance the electrophoretic mobility curves were qualitatively different from those reported by Li and Papkoff.

Preliminary separations on ion exchange columns of the original growth hormone and that treated for 1 hour with alkali showed that the slow component of the alkali-treated hormone corresponded to the unchanged hormone and retained full biological activity. The fast component was somewhat diminished in potency, compared with the untreated hormone.

SUMMARY

Treatment of growth hormone solutions at or below pH 4.0 and at pH 11.5 or above resulted in the formation of progressively increasing amounts, proportional to the time of exposure, of a fast moving electrophoretic component. At both extremes of pH, biological activity diminished with time of exposure and was lost most rapidly in glycine buffer at pH 2.5. In the range from pH 5.0 to 11.5, no electrophoretic change or loss of activity was observed under the conditions employed. Hence the region of maximal stability of growth hormone was determined to be from pH 5.0 to 11.5. Treatment of growth hormone with 0.1 N NaOH gradually converted it to a new electrophoretic component which possessed a higher mobility and thus a higher net charge in both acid and alkaline buffers.

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AMYLO-1,6-GLUCOSIDASE IN MUSCLE TISSUE IN GENERALIZED GLYCOGEN STORAGE DISEASE*

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Previous studies of glycogen storage disease have shown that several distinct types may be recognized (1). In the liver-kidney type, first described by von Gierke, other organs are not affected, the glycogen is normal in structure, and the specific enzymatic lesion consists in low glucose-6-phosphatase activity in liver and kidney cortex (2), the principal tissues which normally contain this enzyme.

In other cases, which are rarer, glycogen storage occurs in all tissues and is especially prominent in heart, diaphragm, and skeletal muscle. Two such cases have recently been analyzed. In one fatal case the structure of the glycogen isolated from heart, skeletal muscle, and liver was normal, and the glucose-6-phosphatase activity of liver and kidney was within the normal range (3). A specific enzymatic lesion in this type of generalized glycogenosis has not so far been detected.

In the other case biopsy specimens of liver and skeletal muscle of a 12 year-old girl were obtained (1). These tissues contained, respectively, 8.7 and 4.6 per cent of a glycogen which had very short outer chains and resembled in this respect the limit dextrin produced from glycogen by the action of phosphorylase.[1] Such an abnormality in structure could result from a deficiency in amylo-1,6-glucosidase (debranching enzyme) which, by splitting the 1,6 linkage at branch points, makes the inner structure of glycogen accessible to phosphorylase action (5). In the absence of this enzyme the action of phosphorylase stops when it approaches the first tier of branch points and thus produces a polysaccharide with short outer chains. An accumulation of this polysaccharide would result because the inner core of glycogen would not be accessible to the degrading action of phosphorylase. Because of lack of material this suggestion could not be tested at that time.

In this paper are reported seven additional cases of glycogen storage in muscle and other tissues, six of them fatal. In five cases glycogen struc-

[1] What is probably another such case, in which, however, only the liver glycogen was analyzed, has recently been reported by Manners (4).

ture was considered to be normal on the basis of branch point percentage and length of outer chains, while in two cases the glycogens had short outer chains. In one of these two (Case 6), glycogens from all tissues investigated had very short outer chains, while in the other (Case 7) the outer chains of liver, kidney, and heart glycogen were much shorter than those of skeletal muscle and diaphragm glycogen.

EXPERIMENTAL

The following methods have been described and will not be detailed here, except where necessary: isolation and purification of glycogen (6), enzymatic determination of end-group and of length of outer chains of glycogen (6), and measurement of glucose-6-phosphatase (2).

In the glucosidase test (7) one uses the phosphorylase limit dextrin of glycogen and inorganic phosphate as substrates and an excess of phosphorylase freed as much as possible of glucosidase as auxiliary enzyme.[2] The products formed (free glucose plus glucose-1-phosphate and, if phosphoglucomutase is present, glucose-6-phosphate) are determined as reducing sugars (after removal of residual polysaccharide and acid hydrolysis of the glucose-1-phosphate). It is essential that a limit dextrin be used as substrate, which has been formed by repeated and exhaustive treatment with phosphorylase. Glycogen, when present, interferes in this test and results in glucosidase values which are too high, because phosphorylase alone can form glucose-1-phosphate from glycogen. This posed a special problem in the case of the muscles of the cases of glycogenosis, with their extremely high glycogen content. In contrast to rabbit muscle, sufficient glycogen was extracted from these human muscles to vitiate a direct determination of glucosidase activity in crude extracts. A partial purification of glucosidase and separation from glycogen were effected as follows.

Weighed portions (1.5 to 10 gm.) of the frozen muscle were allowed to thaw partially and were cut into small pieces with scissors. The muscle was homogenized with 2 volumes of ice-cold water in a micro Waring blendor, and the whole mass was transferred with measured washings to a centrifuge tube. After standing for 10 minutes in the cold, the muscle residue was removed by centrifugation and the supernatant fluid adjusted to pH 6 by addition of a molar solution of sodium acetate (pH 4.6). The precipitate which formed was removed by centrifugation and filtration and the supernatant fluid adjusted to pH 7.0 with solid $KHCO_3$. After

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[2] Up to eleven recrystallizations may be required to free the phosphorylase of the last traces of glucosidase. The same test system, without addition of glucosidase, serves as a phosphorylase blank. In the present work the phosphorylase preparation used gave a negligible blank (about 6 to 10 scale units in the Klett photoelectric colorimeter).

addition of ammonium sulfate solution, pH 7.0, to 0.55 saturation,[3] the sample was kept for 1 hour in an ice bath; it was then centrifuged and the precipitate washed twice with several ml. of 0.55 saturated ammonium sulfate solution in order to remove the glycogen included in the precipitate. The washed precipitate was dissolved in a small volume (0.5 to 1.0 ml., depending on the amount of tissue taken) of a 0.0012 M Versene-0.015 M glycerophosphate buffer, pH 7.2, and dialyzed with stirring for 2 hours in the cold against the same buffer. Protein was determined in the dialyzed solutions by the method of Lowry *et al.* (8). The amount of protein added in the glucosidase tests varied from 0.07 to 0.45 mg. Generally, 0.1 ml. of a 1:2 to 1:10 dilution of the dialyzed glucosidase preparation in 0.0012 M Versene, pH 6.8, was added to the reaction mixture (final volume 1 ml.) which contained 1 mg. of phosphorylase limit dextrin, 50 μmoles of inorganic phosphate, pH 6.8, and 0.2 mg. of repeatedly recrystallized muscle phosphorylase in 0.0012 M Versene, pH 6.8. Glucosidase was added last to start the reaction. After incubation for 20 minutes at 30°, the solution was deproteinized and analyzed for reducing sugars as described previously (7). 1 γ of reducing sugar formed during 10 minutes of incubation under the conditions of this test was designated as 1 glucosidase unit. Enzyme activity in Tables I and II is expressed as units per 100 mg. of muscle.

Several control incubations were carried out with each glucosidase test; in one of these phosphate was replaced by sodium chloride, and this served as the "amylase blank;" in the other no limit dextrin was added, and this served as the "glycogen blank;" phosphorylase alone was also tested to provide a correction in case there were still present traces of glucosidase. The sum of these blanks was applied as a correction and amounted to 10 to 20 per cent of the experimental values when the glucosidase content of the muscle was in the higher range. When the apparent glucosidase activity was very low, the blank values were of special importance in deciding whether or not glucosidase activity was present in a particular muscle. In Table II, where zero activity of glucosidase is recorded, readings in the Klett photoelectric colorimeter varied between 15 and 22 scale units and exceeded the combined blanks by only 1 to 5 scale units, a difference which was regarded as within the experimental error of the method used for the determination of reducing sugars. The low "glycogen blanks" (with two exceptions, when they were 30 and 43 Klett units, respectively) show that the method used resulted in a separation of gluco-

[3] Glucosidase in extracts of frozen human muscle is incompletely precipitated at 0.41 saturation with ammonium sulfate, while nearly complete precipitation is achieved at 0.55 saturation. This is in contrast to fresh rabbit muscle, in which over 80 per cent of the glucosidase is precipitated at 0.41 saturation (7).

sidase from the glycogen present in the crude extracts of the glycogenosis cases. Glucosidase activity was always tested in several dilutions of the enzyme, and fair proportionality was found between dilution and enzyme activity over an 8-fold range of dilution. In the glycogen deposition cases (with the exception of Case 5, in which only 2 gm. of muscle were available) the enzyme was prepared from several aliquots of the same muscle, sometimes on the same day, sometimes after an interval of several weeks, during which the muscle was kept frozen. Activity tests on these extracts were in good agreement, and it should be emphasized that in Cases 6 and 7 no glucosidase was found in several separately prepared extracts.

A method of purification of glucosidase which would be applicable to the quantitative determination of this enzyme in liver tissue has so far not been achieved.

Pertinent clinical information is given at the foot of Tables I and II. All tissues were received packed in dry ice and were kept frozen until analyzed. The time elapsing between death and freezing of the tissues was 4 hours or less, except in Case 3, Table II, when it was 17 hours. In Case 4, Table II, the tissues were in poor condition and histological examination revealed autolysis. Glucosidase activity in the diaphragm of the former and glucose-6-phosphatase activity in the kidney of the latter case were low. It is of interest that the glycogens isolated from these two patients had outer chains of normal length; this would indicate that outer chain length is not significantly affected by postmortem changes in liver, kidney, or muscle.

Results

Table I summarizes the determinations of glucosidase activity in heart and muscle tissues of children who died from causes other than glycogen storage disease. The tissues were frozen within 4 hours after death. Glucosidase activity varied over a considerable range. Whether this variation reflects actual differences in enzyme content or is due to differences in handling the tissues after death before they were received here cannot be decided.

Glucosidase determinations were carried out in three cases of storage disease in which the glycogens had outer chains of normal length (Table II). Cases 3 and 4 showed postmortem autolysis of tissues and were therefore not suitable for enzyme analysis. In Cases 2 and 5 glucosidase activity was within the range seen in children who did not have glycogen deposition disease. In Case 1 low values were obtained.

In Case 5 the percentage of 1,6 bonds and the length of the outer chains of the glycogen fall within the normal range. However, this glycogen was

not completely digested by phosphorylase plus glucosidase, about 20 per cent resisting digestion. Too little glycogen was available for further study. Possibly this case constitutes still another type of glycogen deposition disease. Whether tissues other than skeletal muscle stored glycogen in this subject remains unknown.

In the two cases with abnormal glycogen structure no glucosidase activity could be detected in skeletal or heart muscle. The absence of the glucosidase can explain the abnormal glycogen structure. Measurements of glucosidase activity in liver and kidney could not be carried out, but

TABLE I

Amylo-1,6-glucosidase Activity in Muscles of Infants

Case No.	Age	Muscle	Glucosidase activity
			units per 100 mg.
1	1 day	Diaphragm	157
		Abdominal	150
		Psoas	216
2	2½ mos.	Diaphragm	89
		Psoas	116
3	3 mos.	Thigh	30
		Heart	215
4	Premature birth	Diaphragm	59
		Psoas	61
5	5 mos.	Skeletal	125
Unfrozen rabbit muscle			254

Cases 1, 2, and 4, St. Louis Children's Hospital; Case 2, died of cerebral hemorrhage; Case 3, Jewish Hospital of Brooklyn; diagnosis uncertain; thigh muscle glycogen 0.3, heart 0.53 per cent; Case 5, St. Louis University; diagnosis uncertain; glycogen below 0.1 per cent.

the glycogens isolated from these tissues also had abnormally short outer chains. It seems that this type of general glycogen storage disease, of which four cases are now on record, may be caused by a specific enzymatic lesion; namely, a loss of glucosidase which is presumably normally present in all tissues. As yet, with a very small number of cases studied, there are no family histories to support the view that this form of the disease, like the other forms, is of genetic origin.

Table II shows that glucose-6-phosphatase activity in liver and kidney, which is low in exclusive liver-kidney storage disease, is within the normal range in both types of generalized storage disease. Another difference may be pointed out. While the fat content of the liver is usually very high in fatal cases of liver-kidney storage (9), this is not true in the two

TABLE II

Glycogen Content, Structure, and Enzymes in General Glycogen Storage Disease

In Cases 1 to 4 the glycogen structure was normal; in Cases 6 and 7 the glycogen had abnormally short outer chains; for Case 5, see the text.

Case No.	Tissue	Glycogen content	Fat content	1,6 bonds in glycogen	Degradation of glycogen by phosphorylase	Glucose-6-phosphatase activity	Glucosidase activity
		per cent	*per cent*	*per cent*	*per cent*	$\gamma\, P$ *liberated**	*units per 100 mg.*
1	Heart	7.9		7.8	43		14
	Psoas	12.7		8.1	41		17
	Diaphragm	5.4		8.1	41		13
	Liver	7.0		7.4	39		
	Kidney cortex	1.2					
2	Heart	8.2		7.7	42		102
	Thigh muscle	9.8					83
	" " †	8.3			37		
	Liver	7.5		8.4	40	440	
	Kidney	1.1				420	
3	Heart	10.3		6.2	40		
	Diaphragm	7.0		7.9	37		
	Liver	6.1	8.4				
4	Heart	7.6		8.6	36		
	Diaphragm	12.4		8.3	35		
	Liver	6.6	5.3	7.9	32		
	Kidney cortex	6.0	5.9	8.5	40		
5	Muscle†	13.0		8.8	33		172
6	Heart	4.7		12.0	6		None
	Psoas	3.5		12.6	5		"
	Diaphragm	5.1		12.3	3		"
	Liver	14.2	2.6	9.9	13	350	
	Kidney cortex	0.1	6.0			450	
7	Heart	4.5		12.8	11		None
	Psoas	10.3					"
	Gastrocnemius	14.5		9.2	25		
	Diaphragm	8.9		9.6	26		6
	Liver	8.0	11.3	14.5	10	570	
	Kidney cortex	0.6	4.7	13.8	15	340	

Case 1, male 5 months, St. Louis Children's Hospital; Case 2, male 5 months, Children's Hospital of Philadelphia (Dr. Scott); Case 3, female 3 months, University of Oregon; one sibling well; Case 4, female 3 months, Children's Hospital, New Orleans (Dr. Ordway); three siblings; one died with similar symptoms; Case 5, male 2½ years, Presbyterian Hospital, New York (Dr. Sant'Agnese); normal fasting blood sugar; definite rise after epinephrine, but no rise in blood lactic acid; Case 6, female 13 months, St. Louis Children's Hospital; fasting blood sugar 21 mg. per cent; accidental death; Case 7, female 2 years, University of Colorado (Dr. Alway); normal blood sugar; slight rise after epinephrine.

* Per 100 mg. per 1 hour at 30°.

† Biopsy.

types of generalized storage disease. It may also be noted in Table II that, though glycogen of abnormal structure accumulates in the heart muscle, its concentration does not reach the level found in subjects in which glycogen structure is normal and in which cardiac insufficiency is prominent.

SUMMARY

Determination of the activity of the enzyme amylo-1,6-glucosidase (debranching enzyme) was carried out in skeletal and heart muscle of children. In two cases of generalized storage disease in which the glycogens had abnormally short outer chains, no enzyme activity could be detected. Absence of this enzyme can explain the abnormal glycogen structure and the accumulation of glycogen in the tissues and supports the view that this is a distinct type of glycogen storage disease. In heart and skeletal muscle obtained from three children with general storage disease in which the glycogens had outer branches of normal length, glucosidase activity was within the range found in children who did not have glycogenosis in two instances, while it was very low in the third.

Addendum—Tissues of two additional cases were analyzed while this paper was in print. In both infants the glycogen had normal structure.

Infant, Children's Hospital of Philadelphia (Dr. Wagner). Glycogen content: heart 6.6, psoas 13.0, liver 6.2 per cent. Glucosidase activity: heart 120, psoas 31 units per 100 mg. of tissue.

Infant, Kinderspital Zürich (Dr. Rossi). Glycogen content: heart 5.5, skeletal muscle 13.5, liver 10.6 per cent. Glucosidase activity: heart 72, skeletal muscle 86 units per 100 mg. of tissue.

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RESOLUTION OF FUNGAL CELLULASE BY ZONE
ELECTROPHORESIS

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Results of paper and column chromatography (1, 2) and paper electro-
phoresis (3) of crude preparations of fungal cellulase have pointed toward
the multiple nature of this system. Results of ultracentrifugation and
moving boundary electrophoresis of fractionated cellulase have, on the
other hand, suggested its unienzymatic nature (4). In the present study,
zone electrophoresis over extended distances has been utilized to provide
more critical data bearing on this question. The findings support the
hypothesis of a multiple enzyme system.

Materials and Methods

Enzyme Preparations—Cellulase solutions were prepared in about 25 liter
lots by growing *Myrothecium verrucaria* QM 460 on a 0.4 per cent suspen-
sion of filter paper in 12 gallon carboys at room temperature for 2 weeks.
The medium used was that of Reese, Siu, and Levinson (5). Aeration was
provided at the rate of about 25 liters per minute. Special precautions
were taken to avoid contamination. Changes in protein, activity, and pH
in filtrates of a typical culture during the incubation are shown in Fig. 1.
It will be noted that the production of protein was parallel with that of
activity. The unit of activity is defined later.

Following filtration through cheese-cloth and clarification in a Sharples
centrifuge, the enzyme-containing medium was concentrated at 5° in a
pressure ultrafilter (6), dialyzed in collodion bags against distilled water
until freed of salts, and lyophilized to yield the crude enzyme preparations
used for electrophoretic studies. The final recovery of enzyme activity
approached 80 per cent. On the average, the preparations contained equal
amounts of protein and carbohydrate, and the activity was about 24 units
per mg. of total solids. The carbohydrate was found by chromatographic
tests to contain mannose and galactose but no glucose.[1] Its origin is
unknown.

Zone Electrophoresis—Zone electrophoresis was carried out with potato

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[1] The chromatographic tests were made by Dr. Elwyn T. Reese and Mr. William
Gilligan.

starch[2] as the supporting medium in an apparatus similar to that of Kunkel and Slater (7).   The starch block was favored over paper electrophoresis because of the smaller likelihood of adsorption and of hydrolytic action of the enzyme on the starch.   pH changes at the electrodes were avoided by circulating the contents of the outside electrode vessels with the aid of a pump,[3] the fluid being drawn from the cathode vessel into the anode vessel and then allowed to return by way of a siphon.   To prevent accumulation in the siphon of gas from the electrodes, the siphon was mounted as an inverted V, at the top of which a tube was attached for collecting the gas.
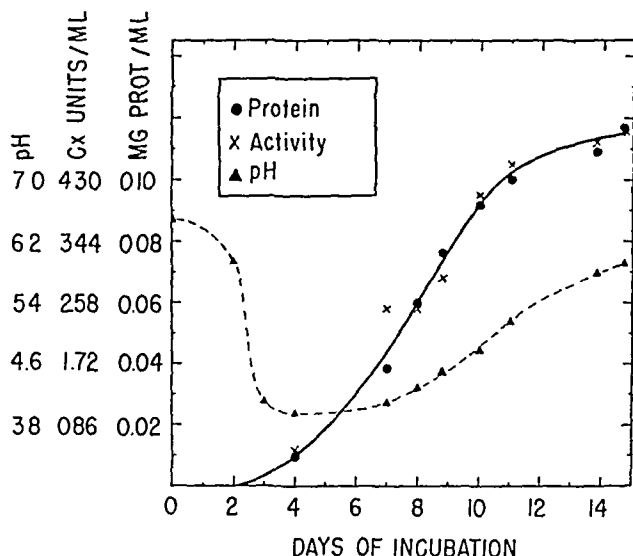
FIG. 1. Production of protein and cellulase and changes in pH in filtrates of *M. verrucaria* growing on cellulose.

The runs were made at 5° in buffers of 0.05 ionic strength, principally in sodium phosphate at pH 7.   The starting sample, amounting to 100 mg. of crude enzyme in 2 ml. of buffer, was introduced into a 4 cm. slit cut crosswise in a 50 × 10 × 1.3 cm. block of the starch.   The slit was located near the anode end of the block to compensate for the electroosmotic flow of buffer solution toward the cathode.

Quantitative recovery was sacrificed for improved resolution of the components by discarding strips 2 cm. wide from each of the long sides of the block at the completion of each run.   This procedure reduced the over-

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[2] Chromatographic potato starch sold by the Amend Drug and Chemical Company, Inc., New York, was found to be more satisfactory than certain other commercial preparations tested because of its low content of soluble reducing sugar. The starch was not washed before use.

[3] A pump sold by Sigmamotor, Inc., Middleport, New York, was found to be particularly useful for this purpose, since it avoided direct contact of the fluid being pumped with the pump parts.

lapping that results from the observed curvature of the zones of the components. The remaining (center) strip of the block was then cut crosswise into sections 1 cm. wide, each of which was extracted with 20 ml. of phosphate buffer, 0.05 ionic, pH 7. Finally, analyses for protein, carbohydrate, and activity were made on suitable aliquots of the extracts.

Analyses[4]—Protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall (8). Bovine plasma albumin was employed as a standard. The results agreed with those obtained by the micro-Kjeldahl method (9).

Carbohydrate analyses were carried out by the method of Rimington (10), with use of glucose as the standard.

Enzyme activities were determined as "Cx units" by the method of Reese and coworkers (1, 2, 5). The term Cx is used (5) to designate the particular enzyme system which hydrolyzes the $1,4$-β-glucosidic linkage as found in cellulose and as measured by the amount of reducing sugars obtained by enzymatic hydrolysis of carboxymethyl cellulose. The Cx unit is defined (2) as the amount of enzyme in 10 ml. of assay medium (0.5 per cent Hercules carboxymethyl cellulose No. 50T in 0.05 M citrate at pH 5.4) required to give a reducing value as glucose of 0.40 mg. per ml. in an hour at $50°$.

Results

Electrophoretic patterns of individual preparations of crude enzyme were found to be comparable in the number and relative positions of observed active components. It appeared justified, therefore, to pool all of the preparations in order to have available a larger, more completely uniform stock of the enzyme.

Results obtained with the pooled enzyme, when this was subjected to electrophoresis in phosphate buffer at pH 7 for 42 hours at 400 volts, are presented in Fig. 2. The pattern of distribution of enzyme activity indicates the presence of at least eight distinct enzyme components, one of which was completely separated from the others. The pattern was closely confirmed in a duplicate experiment. In control runs with plasma albumin and hemoglobin, it was found that migration under the conditions used with the enzyme was normal and undisturbed and, further, that complete resolution of the albumin and hemoglobin was readily obtained in tests with mixtures of the two proteins.

It appeared that a more complete resolution of the enzyme components should be possible if the time of electrophoresis were extended still further. This was accomplished, without lengthening the block, by making a run similar to the above, removing the anode section of the block up to and

[4] Mr. Alfred Sunseri assisted with the analyses.

including Component I, shifting the remaining cathode end of the block toward the anode, and attaching a fresh starch block to the incomplete cathode end. After electrophoresis was continued for an additional 40 hours at 400 volts, the distribution of components was that shown in Fig.
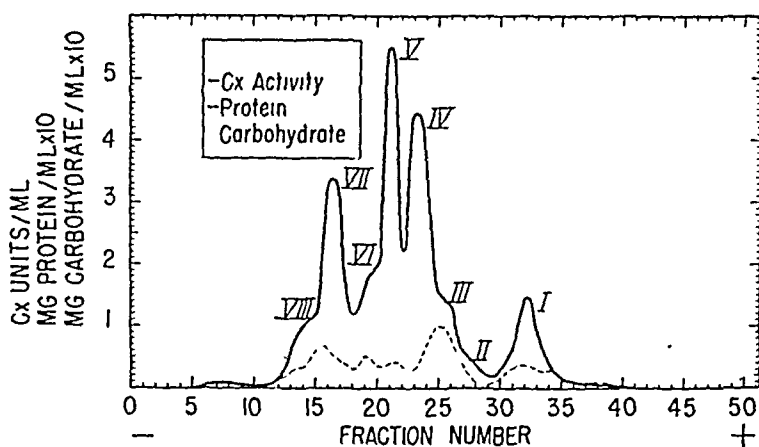

FIG. 2. Distribution of protein, carbohydrate, and activity in zone electrophoresis of crude cellulase at pH 7 for 42 hours at 400 volts.
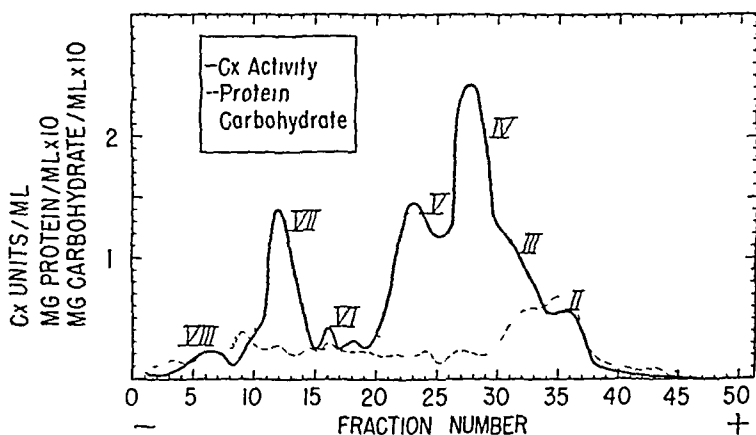

FIG. 3. Distribution of protein, carbohydrate, and activity in zone electrophoresis of crude cellulase at pH 7 for 40 hours at 400 volts, following preliminary electrophoresis at pH 7 for 42 hours at 400 volts and removal of Component I contained in anode end of block.

3. Here it may be noted that Components VII and VIII have now been practically completely separated from the others. The presence of Component I in the anode section removed earlier in the run is shown by the distribution pattern of Fig. 4.

Further electrophoretic studies showed that the resolution of the components was not improved when tested in Veronal and in glycine-NaOH buffers at pH levels up to 10, and that it was much poorer when tested in acetate and in glycine-HCl buffers at pH levels down to 3. The distribu-

tion of protein and carbohydrate at pH 3 (Fig. 5) was of particular significance, however, in that it demonstrated that only a fraction of the total protein in these preparations, and probably none of the carbohydrate, was associated with cellulase activity.
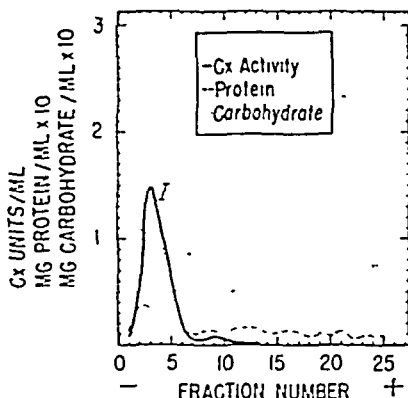

FIG. 4. Distribution of protein, carbohydrate, and activity in anode end of block after zone electrophoresis at pH 7 for 40 hours at 400 volts.
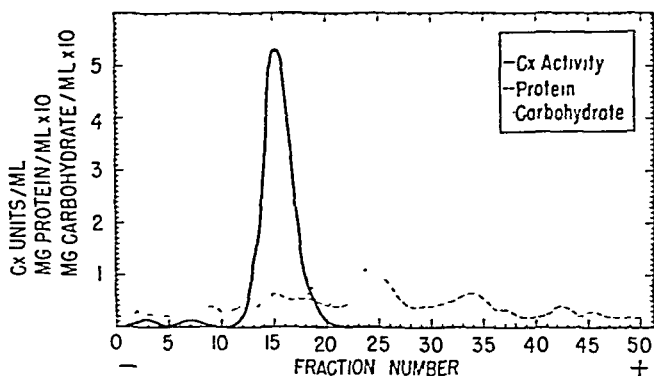

FIG 5. Distribution of protein, carbohydrate, and activity in zone electrophoresis of crude cellulase at pH 3 for 12 hours at 400 volts

The total recoveries of protein, carbohydrate, and activity in typical runs at different pH values are presented in the first part of Table I. Recoveries in controls, in which various mixtures of enzyme, buffer, and starch were gently agitated overnight without being subjected to electrophoresis, are also shown. The proportions of the enzyme, buffer, and starch in the controls were the same as those used in the extraction of the starch block sections. It may be seen from Table I that in the electrophoresis runs the recoveries of protein and activity were somewhat low, while those of carbohydrate were quite high.

The possibility of loss of protein and activity due to adsorption on the

starch appears to be ruled out by the fact that in the controls the recoveries of these factors were not lower in the presence of starch than in its absence. The losses were, therefore, undoubtedly largely the result of discarding the 2 cm. strips from the sides of the blocks, as mentioned earlier, although in the runs at pH 3 the apparent loss in protein is partially attributable to an effect of glycine buffer of pH 3 on the analyses. The greater losses in activity in runs at pH 3 and pH 10 than at pH 7 may be attributed to inactivation, as demonstrated by the controls and as would be expected from previous work (11, 12). The fact that at pH 7 the recovery of activity

TABLE I

Per Cent Recovery of Protein, Carbohydrate, and Activity in Electrophoresis Runs and in Controls

Description of test	Protein	Carbohydrate	Activity
Electrophoresis run, pH 3	74	160	25
" " " 7..	79	152	38
" " " 10	80	153	27
Control, enzyme + buffer + starch, pH 3 .	60	223	65
" " + " + " " 7	103	269	109
" " + " + " " 10 .	97	171	71
" " + " pH 3 .	70	91	77
" " + " " 7 . .	90	108	94
" " + " " 10 . .	91	97	9
" buffer + starch, pH 3		14*	
" " + " " 7		24*	
" " + " " 10		18*	

* These values are calculated on the basis of the amount of carbohydrate which would have been contributed by the enzyme used in the other controls had the enzyme been present without acting on the starch.

was lower than that of protein may be explained by a synergistic effect (2), whereby the different enzymes can show less activity when separated from one another than when in mixtures.

The results of the controls of starch plus buffer show that the high carbohydrate values obtained in the electrophoresis runs could have been only partially due to the presence of soluble reducing sugars in the starch used as the supporting medium. It may be concluded, therefore, that the high carbohydrate values must have been due primarily to the presence of amylase in the preparations.

DISCUSSION

The observed separation of active enzyme components does not appear to be the result of disturbances such as convection during electrophoresis,

since the conditions were found to be entirely suitable when known proteins were tested. The findings lend strong support, therefore, to the hypothesis of the multiple nature of the cellulase system.

The most probable reason for the reported failure to demonstrate multiple components by moving boundary electrophoresis (4) is the use of too short distances of migration. The spread of the enzyme reported with the moving boundary method was only about one-fortieth that attained with the starch block. Less likely is the possibility that the single component resulted from a difference in conditions under which the enzyme was elaborated by the fungus. Also less likely is the possibility of fractionation of the enzyme during the purification steps, since there appears to be no reason to expect that the particular precipitating agents used should succeed in effecting a sharp separation of closely related enzyme proteins.

Final proof for the multiplicity of enzymes in the cellulase system must await their complete separation from one another and from inactive contaminants. The use of zone electrophoresis, particularly preparative designs (13–15), is clearly indicated for this purpose, although it will probably need to be supplemented by chromatographic (2, 3, 16), precipitation (4), and other methods. Comparison of the isolated enzyme components with respect to activity per unit weight, substrate specificity, physical properties, amino acid composition, and other properties will be of particular interest and importance.

SUMMARY

Zone electrophoresis of crude fungal cellulase over extended distances has indicated the multiple nature of the enzyme system.

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THE METABOLISM OF MUCOPOLYSACCHARIDES IN ANIMALS

III. FURTHER STUDIES ON SKIN UTILIZING C14-GLUCOSE, C14-ACETATE, AND S35-SODIUM SULFATE*

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A method for the isolation of an analytically pure hyaluronic acid (HA) and chondroitinsulfuric acid (CSA) fraction from the skin of rabbits has been described (1). The method was applied to a study of the half life times from decay curves after the injection of C14-labeled acetate (2). The half life times for HA and for CSA in the skin of rabbits were found to be 1.9 and 7 days, respectively.

In experiments utilizing C14-labeled acetate, most of the radioactivity in the HA and CSA fractions was found in the N-acetyl component (2). It is conceivable that this portion of the molecule turns over at a different rate from the others. It was of interest, therefore, to study the half life times of the skin mucopolysaccharides utilizing precursors of other portions of the molecule. Since it appeared desirable to obtain information concerning the metabolism of mucopolysaccharides in species other than the rabbit, some experiments were performed on the rat. Analytical data for the HA and CSA from the skin of rats are comparable[1] to those previously obtained for these substances isolated from the skin of rabbits (1).

Previous studies have shown that glucose serves as a precursor for the glucosamine (3, 4) and the glucuronic acid (5) portions of the HA synthesized by a group A streptococcus, and also as a precursor for glucosamine in the serum of rats (6). That inorganic sulfate is incorporated into the CSA of skin has also been demonstrated (7). C14-labeled acetate or glucose was, therefore, administered simultaneously with $Na_2S^{35}O_4$ to rats and rabbits. The results indicate that, in the HA molecule, the glucosamine and N-acetyl components turn over at the same rate. In the CSA molecule, galactosamine, N-acetyl, and sulfate are also metabolized at the same rate, but more slowly than the moieties of HA.

EXPERIMENTAL

Animals and Materials—The animals were maintained on Rockland chow and were allowed food and water *ad libitum* throughout the experimental period. The injection schedules were identical for both groups of experiments, the total radioactive material being administered subcutaneously in three injections, once every 4 hours.

In the first experiment adult male rabbits of the Swift strain, weighing between 2.5 and 3.2 kilos, were used. Each of eight rabbits received 49 μc. of C^{14} uniformly labeled glucose and 1.2 mc. of $Na_2S^{35}O_4$ as an isotonic mixture of the two substances. One pair of rabbits was sacrificed 16 hours after the last injection. The remaining six rabbits were killed in pairs, 4, 8, and 16 days after the first injection.

60 adult male rats of the Sprague-Dawley strain were used in the second experiment. Each rat was injected with 57 μc. of C^{14}-carboxyl-labeled acetate and 15 μc. of $Na_2S^{35}O_4$ as an isotonic mixture. The animals were sacrificed in groups of twelve at 1, 3, 5, 9, and 17 days after the first injection. Each group weighed between 3.0 and 3.9 kilos.

C^{14} Uniformly Labeled Glucose—The radioactive glucose was prepared from a sample of C^{14} uniformly labeled sucrose[2] which had a radioactivity of 218 μc. per gm. The sucrose was hydrolyzed with 0.1 N H_2SO_4 and, after neutralizing with $BaCO_3$, the glucose was isolated from the hydrolysate by chromatography on a column of powdered cellulose with *n*-butanol-ethanol-water (10:3:6) as developing agent.

Methods

The HA and CSA fractions were isolated from the skin by a method described in a previous publication (1).

The hexosamines were isolated from HA and CSA by a modification of the method of Gardell (8), as described by Dorfman *et al.* (9).

The separated polysaccharides and their respective hexosamines were oxidized to CO_2 and the C^{14} counted as $BaCO_3$ in an internal gas flow counter, corrected to "infinite thickness." A silver wire was used in the combustion tube filling to assure the complete removal of the S^{35} during the oxidation of the CSA. Appropriate control experiments showed no contamination of C^{14} samples with S^{35}.

The radioactivity of the S^{35} in the CSA fractions was counted as $BaSO_4$, corrected to "infinite thickness." Precipitation of the $BaSO_4$ was carried out after hydrolysis of the sulfated polysaccharide with 4 N HCl for 8 hours and dilution with 0.03 to 0.04 mmole of inactive sulfate.

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[2] We are grateful to Dr. Norbert J. Scully of the Argonne National Laboratory for the uniformly labeled sucrose used in these experiments.

Results

A plot of the C^{14} of the HA and CSA fractions of the skin at various time intervals after the simultaneous injection of C^{14} uniformly labeled glucose
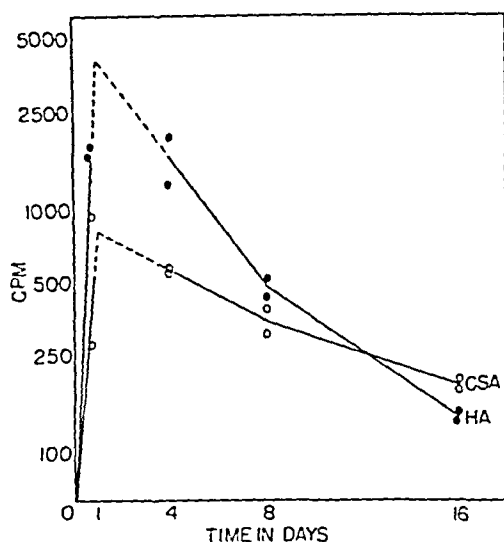

Fig. 1. A plot of the C^{14} of the HA and CSA of the skin of rabbits (corrected for body weight) injected with C^{14}-glucose and $Na_2S^{35}O_4$. Zero day on the abscissa indicates the time of the last injection. The ordinate represents the log of the radioactivity in counts per minute, corrected to "infinite thickness." Maximal radioactivity was obtained by extrapolation, as described in the text.

TABLE I

Comparison of Half Life Times of Skin Mucopolysaccharides and Some of Their Components

The figures represent days.

Species	Hyaluronic acid (HA)			Chondroitinsulfuric acid (CSA)			
	C^{14}-HA	N-Acetyl	Glucos-amine	C^{14}-CSA	N-Acetyl	Galactos-amine	S^{35}
After C^{14}-acetate and $Na_2S^{35}O_4$							
Rabbits*	2.4	2.7		7.6	5.3 (?)		
Rat	4.5			8.1			10.7
After C^{14}-glucose and $Na_2S^{35}O_4$							
Rabbit	3.7		3.4	7.7		7.4	10.0

* The data from this experiment have been published (2) and are used here for comparative purposes.

and $Na_2S^{35}O_4$ to rabbits is given in Fig. 1. It was apparent that the 16 hour interval permitted before sacrifice of the first group of animals was not sufficient to obtain maximal labeling. This interval had been found adequate in previous experiments following acetate administration. The difference is probably due to the slower absorption of glucose. The
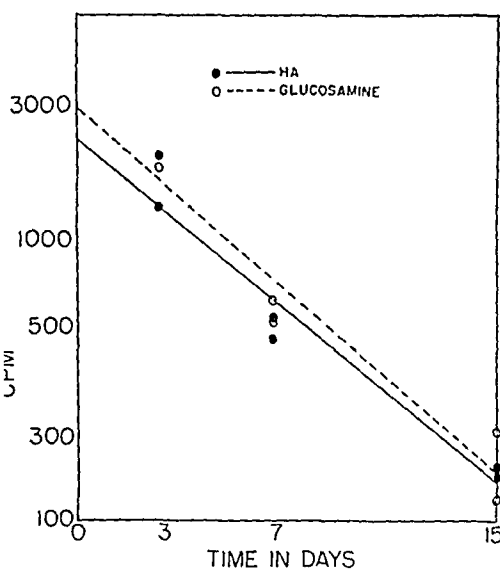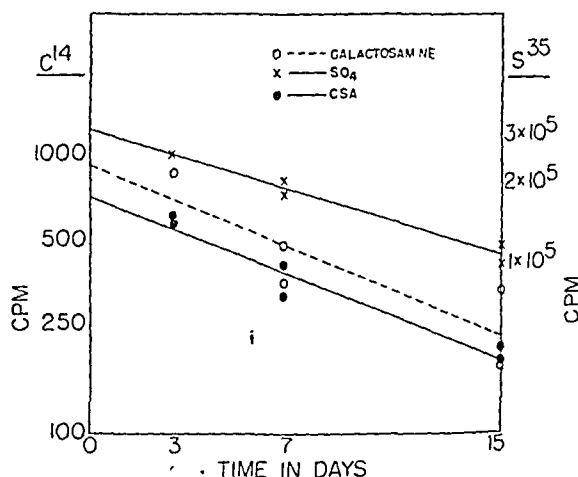

Fig. 2   Fig. 3

FIG. 2. A semilogarithmic plot of the C^{14} of glucosamine compared with that of the HA isolated from the skin of rabbits injected with C^{14}-glucose and $Na_2S^{35}O_4$. The data are plotted by a line of best fit calculated by the method of least squares from the values obtained at 3, 7, and 15 days. The values for maximal labeling were obtained by extending the line to the ordinate. The C^{14} was measured as $BaCO_3$, corrected to "infinite thickness."

FIG. 3. A comparison of the decay curves for the C^{14} of galactosamine and CSA and for the S^{35} of the CSA isolated from the skin of rabbits injected with C^{14}-glucose and $Na_2S^{35}O_4$. The data are plotted by a line of best fit calculated by the method of least squares from the values obtained at 3, 7, and 15 days. The values for maximal labeling were obtained by extrapolating to zero time. The C^{14} and the S^{35} were measured as $BaCO_3$ and $BaSO_4$, respectively, corrected to "infinite thickness."

activity at the time of maximal labeling, therefore, was obtained from the intersection of the uptake curve and the decay curve. Despite the departure from linearity, the data depicted in Fig. 1 demonstrate that the C^{14} is lost from the HA more rapidly than from the CSA. The half life times, as calculated from the line of best fit, are 3.7 days for HA and 7.7 days for CSA (Table I).

The hexosamines isolated from hydrolysates of the HA and CSA fractions contained approximately the same C^{14} concentration as the whole molecule at each time interval. This is demonstrated in Figs. 2 and 3,

where the radioactivity of the amino sugars is compared with that of the respective acid mucopolysaccharides when the data are plotted by a line calculated by the method of least squares.

A close parallelism was found between the rates of CSA decay as determined by S^{35} and by C^{14} (Fig. 3). The half life time of the CSA calculated from the curves was found to be 7.7 and 10.0 days, respectively, on the basis of the C^{14} and S^{35} decay rates (Table I).

The results in rats which received C^{14}-carboxyl-labeled acetate (Fig. 4) were similar to those found in rabbits. Furthermore, no essential differ-
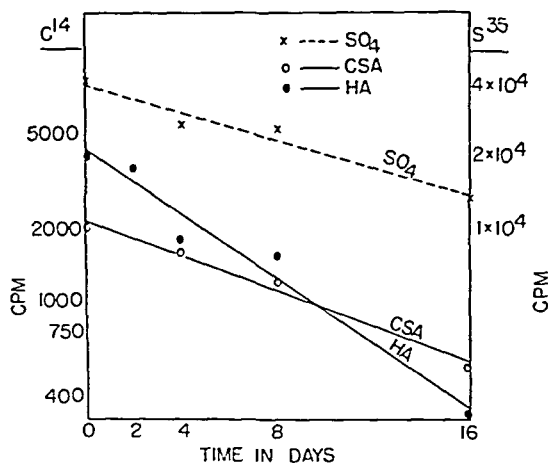

FIG. 4. A semilogarithmic plot of the C^{14} of the HA and CSA and of the S^{35} of the CSA isolated from the skin of rats injected with C^{14}-carboxyl-labeled acetate and $Na_2S^{35}O_4$. Each point represents the value obtained from a pool of twelve rat skins. The data are plotted by the method of least squares. The C^{14} and the S^{35} were measured as $BaCO_3$ and $BaSO_4$, respectively, corrected to "infinite thickness."

ence was demonstrable in the half life times of the skin HA and CSA of rats after the simultaneous administration of C^{14}-acetate and $Na_2S^{35}O_4$ as compared with the skin mucopolysaccharides of rabbits after C^{14}-glucose plus $Na_2S^{35}O_4$ administration (Table I).

DISCUSSION

The values of 3.7 and 7.7 days obtained for the half life times of the HA and the CSA, respectively, from the skin of rabbits injected with C^{14} uniformly labeled glucose are in close agreement with our previous finding in animals injected with C^{14}-labeled acetate (2). The half life times reported in the earlier paper were estimated graphically from a line drawn through the experimental points as the time when half the maximal radioactivity was attained. For purposes of comparison, a line of best

fit was calculated for the first three points of the decay curve so that the half life times of the two mucopolysaccharides and their *N*-acetyl component could be compared with the data obtained in the present study (Table I).   The somewhat lower value for the half life time of the *N*-acetyl as compared with that of the CSA may have been due to a high dilution with carrier sodium acetate and to the low counts above background.

The rates of mucopolysaccharide synthesis in the skin of the rat appear to be similar to those in the skin of the rabbit.   The half life times of 10.0 days (rabbit) and 10.7 days (rat) found for the skin CSA as determined by S^{35} agrees with the value of 8 to 9 days obtained by Boström and Gardell (7) for this fraction in the skin of rats injected with $Na_2S^{35}O_4$.

The interpretation of the data on the CSA fraction is proposed with reservation, since the CSA as isolated in its present form appears to be a mixture of sulfated polysaccharides (1), each of which may have entirely different rates of synthesis.   Two components of rabbit skin CSA have been separated by alcohol fractionation.[1]   One of these components is readily attacked by testicular hyaluronidase while the other is unaffected by the enzyme.   Fractionation of the radioactive CSA samples obtained in the present experiments was not attempted because of insufficient material.

In our experiments with C^{14} uniformly labeled glucose the radioactivity of the CO_2 liberated by decarboxylation of the uronic acid was too low for accurate counting.   However, since the C^{14} concentration of the whole molecule and that of the hexosamines were identical, we can infer that the C^{14} concentration of the uronic acid was likewise identical.

SUMMARY

1. The rates of mucopolysaccharide synthesis were determined in the skin of rabbits injected simultaneously with C^{14} uniformly labeled glucose and $Na_2S^{35}O_4$, and were found to agree with those of rats similarly injected with C^{14}-carboxyl-labeled acetate and $Na_2S^{35}O_4$.

2. From the half life times it would appear that the glucosamine and *N*-acetyl moieties of the hyaluronic acid in the skin of rabbits turn over at the same rate.   While the components of the chondroitinsulfuric acid fraction are metabolized at a slower rate than those of the hyaluronic acid, they also appear to turn over at the same rate.

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PURINE FERMENTATION BY CLOSTRIDIUM CYLINDROSPORUM

I. TRACER EXPERIMENTS ON THE FERMENTATION OF GUANINE*

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The isolation of two anaerobic bacteria, *Clostridium acidi-urici* and *Clostridium cylindrosporum*, capable of fermenting various purines was described by Barker and Beck in 1942 (1). The organisms were initially differentiated by their morphological characteristics. Ammonia, carbon dioxide, and acetic acid were identified as products of the fermentation of uric acid, xanthine, hypoxanthine, or guanine by *C. acidi-urici* (2). Glycine was identified as an additional fermentation product formed by *C. cylindrosporum* (2), and considerable evidence was obtained which implicated glycine as an intermediate in the fermentation of purines by *C. acidi-urici*. A close physiological relationship between the two organisms was therefore evident.

Karlsson and Barker (3) prepared C^{14}-labeled samples of uric acid by feeding formate-C^{14}, carbonate-C^{14}, or glycine-C^{14} to pigeons. The uric acid obtained in this manner, while not altogether specifically labeled, was used to determine the purine carbon sources of the carbon dioxide and acetic acid in the *C. acidi-urici* fermentation (4). It was found that carbon dioxide was mainly derived from C-2+8 and C-4; the methyl of acetic acid from C-2+8, C-5, and carbon dioxide; and the carboxyl of acetic acid from C-5, the methylene of glycine, and carbon dioxide.

The present investigation was undertaken to determine the carbon and nitrogen sources of glycine in this fermentation. Since glycine is accumulated as a fermentation product by *C. cylindrosporum*, it seemed desirable to use this organism, rather than *C. acidi-urici*, for these studies. During the course of the investigation, formic acid was identified as a product of the fermentation of guanine by *C. cylindrosporum*, and its carbon sources

together with those of the other products, carbon dioxide, glycine, and acetic acid, were also determined.

Materials

Guanine-2-C^{14} was purchased from the Southern Research Institute. Guanine-4-C^{14} was a gift of Dr. E. L. Bennett (5). Guanine-6-C^{14} and guanine-8-C^{14} were synthesized by Dr. R. G. Bartsch (5). Uric acid-5-C^{14} was the biosynthetic sample prepared by Karlsson and Barker (3). In a more recent determination by Dr. A. Weissbach and Dr. D. B. Sprinson of the distribution of C^{14} in the sample (6), 70 per cent of the C^{14} was found in C-5 compared to 52 per cent previously reported. Carbon atoms 2 and 8 contained 27.5 per cent; C-6 contained 1.5 per cent and C-4 only 0.9 per cent. Uric acid-7-N^{15}, containing 0.12 atom per cent excess N^{15} and isolated from the urine of subjects fed glycine-N^{15}, was a gift of Dr. D. Stetten. C^{14}-labeled formic acid, methylene- or carboxyl-labeled glycine, and methyl of carboxyl-labeled acetic acid were synthetic materials. Xanthine-8-C^{14} and xanthine-2-C^{14} were prepared from the corresponding guanine-C^{14} samples by treatment with nitrous acid (7). The product was purified by chromatography on paper in the isoamyl alcohol-phosphate system (pH 7) of Carter (8), and diluted with unlabeled xanthine.

Methods

Cultures—Stock cultures of *C. cylindrosporum*, strain HC1, and *C. acidi-urici*, strain 9a, were grown as stabs on a medium of the following composition, in mg. per 100 ml. (Medium 1): uric acid 150, $MgSO_4 \cdot 7H_2O$ 5, $FeSO_4 \cdot 7H_2O$ 0.25, $CaCl_2 \cdot 7H_2O$ 0.6, $K_2HPO_4 \cdot 3H_2O$ 140, Difco yeast extract 100, sodium thioglycolate 50, agar 2000. The medium was adjusted to pH 7.4 to 7.6 with KOH, and a small amount of methylene blue was added. The tubes were sealed with pyrogallol-potassium carbonate and grown at 37°. The cultures were stored at 4°.

Tracer experiments were carried out on media of the following composition, in mg. per 100 ml. (Medium 2): guanine 151, uric acid 16.8,[1] Difco yeast extract 25, sodium thioglycolate 50, $CaSO_4$ 0.6, $FeSO_4 \cdot 7H_2O$ 0.25, $K_2HPO_4 \cdot 3H_2O$ 140, $MgSO_4 \cdot 7H_2O$ 5, sodium carbonate 1060. Phenol red was added as a pH indicator. The medium, without carbonate, was adjusted to pH 7.4 to 7.6 and autoclaved in a 100 ml. volumetric flask. After cooling, the sodium carbonate was added as a sterile solution, and the medium was adjusted to pH 7.4 to 7.6 with a sterile solution of 1 N sulfuric acid and diluted to volume. Additional supplements were added in

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[1] The organism grows much more slowly on guanine than on uric acid (9). A small amount of uric acid was therefore included in the medium to insure the rapid initiation of growth.

particular experiments indicated in the experimental section at the following levels, in mg. per 100 ml.: sodium acetate 410, sodium formate 340, or glycine 150. A 5 ml. aliquot was removed for determination of initial concentrations. The flask was then inoculated with 2 drops of a 16 to 24 hour culture and made anaerobic with Oxsorbent (Burrell Corporation, Pittsburgh). The inoculated medium was incubated at 37° for 5 to 7 days (a day or two longer than necessary for disappearance of the precipitated uric acid).

For preparation of cell-free extracts, the organism was grown on Medium 1 without added agar. The cells were harvested by centrifugation, washed with cold 0.4 per cent KCl which had been previously boiled to remove dissolved oxygen, and lyophilized. The extract was prepared by incubating 500 mg. of lyophilized cells with 20 ml. of 0.1 M potassium phosphate buffer, pH 7.0, 0.01 M with respect to cysteine in an evacuated vessel at 35° for 1 hour. The incubation mixture was centrifuged at 0° and 25,000 × g for 10 minutes. To reduce ultraviolet-absorbing material, the extract was saturated with ammonium sulfate and the precipitated protein was redissolved in 0.1 M buffer-0.01 M cysteine solution and stored in an evacuated tube at −10°.

Quantitative Determinations of Fermentation Products—Carbon dioxide was determined by the manometric method of Peters and Van Slyke (10). Ammonia was steam-distilled from sodium tetraborate, collected in boric acid, and titrated with acid. Glycine was determined by the method of Alexander *et al.* (11). Formic and acetic acids were the only acids detected on silica gel column chromatography of the volatile acid fraction (12). These acids were determined by a modification of the method of Friedemann (13). In this method, total steam volatile acids are determined by titration, acetic acid is determined by Duclaux distillation from a mixture containing $MgSO_4$ and $HgSO_4$, and formic acid is calculated as the difference between total steam-volatile acids and the amount of acetic acid found. The formation of formic acid among the fermentation products was also demonstrated by the manometric method of Pirie (14) and the colorimetric method of Grant (15). However, the titrimetric method was found to be more convenient for routine analyses than either of the other two. The extent of purine decomposition was determined from measurements of the optical density of the fermented medium at 260 mμ.

Isolation and Degradation of Fermentation Products—Carbon dioxide was liberated from an aliquot of the fermentation medium with concentrated lactic acid and was collected as barium carbonate with an evacuated diffusion apparatus (16). The barium carbonate was well washed with boiling water, suspended in 95 per cent ethanol, plated on aluminum disks, dried under an infra-red lamp, and weighed. The C^{14} was determined with

a thin window Geiger counter, and the results were calculated to zero sample thickness.

The remainder of the fermentation medium was centrifuged to remove cells and precipitated salts.   Glycine (120 mg.) was added to the measured volume of medium to give an approximate 5-fold dilution of the glycine formed in the fermentation.   The medium was made alkaline to phenol red with potassium hydroxide and evaporated to dryness on a steam table. The residue was suspended in 10 to 15 ml. of water, adjusted to pH <1.0 with sulfuric acid, and steam-distilled, the volatile acids being collected in 500 ml. of distillate.   After neutralization with sodium hydroxide, this fraction was concentrated on a steam bath and diluted to 25 ml. The non-volatile fraction was recovered for isolation of glycine.

Formic acid was degraded to carbon dioxide by heating it with mercuric sulfate and was collected as barium carbonate.   The reaction was carried out in a diffusion apparatus placed in an Arnold steamer for 30 minutes.

Acetic acid was distilled from the remainder of the volatile acid fraction after it had been treated with mercuric sulfate to destroy the formic acid present.   The acetic acid was degraded by pyrolysis of the barium salt (17).

Glycine was recovered from the non-volatile fraction after reaction with benzoyl chloride and recrystallization of the resultant hippuric acid. In experiments with uric acid-N^{15} the hippuric acid was isolated after chromatography on a silica gel column described for the separation of volatile acids (12).

Glycine, obtained on alkaline hydrolysis of the hippuric acid, was degraded to carbon dioxide and formaldehyde by treatment with ninhydrin in a diffusion apparatus.   Formaldehyde was oxidized to formic acid and then to carbon dioxide, as described by Sakami (18).   The C^{14} content of the guanine used was determined directly on samples weighing less than 1 mg. N^{15} was estimated by means of a Consolidated-Nier mass spectrometer by Dr. C. C. Delwiche and Dr. J. Weijler.

Results

Fermentation Products and Balances—A sample of uric acid-5-C^{14} and the four synthetic samples of guanine, each labeled with C^{14} in a different atom, were fermented under the conditions described under "Methods." Unlabeled guanine was also fermented in the presence of added pools of C^{14}-labeled samples of the fermentation products.   The fermentation balances are given in Table I.   The C^{14} recovered in each product is reported in Table II.   The average carbon, nitrogen, and C^{14} recoveries in these thirteen fermentations were 93.4 $\pm$ 2.2,[2] 93.4 $\pm$ 1.2, and 95.4 $\pm$ 2.9

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[2] Standard deviation.

per cent, respectively. The fermentation of guanine by *C. cylindrosporum* may be summarized by the following equation, based upon the average recovery of products obtained in Experiments 1 and 7 to 10.

1 guanine → 4.8 ammonia + 2.8 CO_2 + 0.33 glycine

$$+ \ 0.99 \ \text{formic acid} + 0.38 \ \text{acetic acid}$$
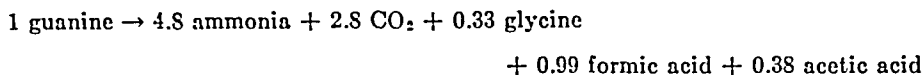
Fermentations carried out in the presence of pools of formic acid (Experiment 2) or glycine (Experiments 5 and 6) did not differ significantly (Table I). However, in the presence of a pool of acetic acid (Experiments 3 and 4), the formation of acetic acid appears to have been suppressed. The apparent utilization of acetate in Experiment 3 is probably an error, since no appreciable amount of C^{14} was recovered in other products. The same products are formed from uric acid and from guanine by this organism (Experiment 11). However, the yields of formic acid and glycine from uric acid are only about half of those from guanine.

The fermentation of guanine by *C. acidi-urici* was determined in Experiments 12 and 13. A pool of added glycine was present during the fermentation. Under these conditions, glycine is partially utilized by this organism. The major volatile acid accumulated is acetic acid, and only small amounts of formic acid could be detected.

Purine Fermentations in Presence of C^{14}-Labeled Products—The distribution of C^{14} among each of the product carbon atoms during a fermentation and the per cent of the administered C^{14} recovered in each of the carbon atoms of the products is given in Table II.

The utilization of carbon dioxide was determined in Experiment 1. The fermentation was carried out in the presence of 3.2 times as much carbon dioxide as was formed from the fermented substrates, and the specific activity of the final carbon dioxide was 0.85 times that initially present. An extensive conversion of carbon dioxide to formic acid was observed. This reaction complicated the interpretation of the results. Experiments (Table III) were designed to determine whether the effects of this interconversion could be minimized by using shorter incubation times. However, it was found that the ratio of C^{14} in carbon dioxide and formic acid is almost constant at 28, 32, or 180 hours, when 59, 74, or 100 per cent, respectively, of guanine-8-C^{14} had been fermented. It was possible to obtain extracts of the organism which decomposed xanthine but were free of formic dehydrogenase, the enzyme probably responsible for the observed interconversion of formic acid and carbon dioxide. The results obtained with such an extract will be discussed in a later section. Acetic acid and glycine were both labeled by the carbon dioxide. The methyl of acetic acid had 5.2 times the activity of the carboxyl group, which is in good agreement with the value of 5.8 found for this ratio by Barker and

Elsden (19) with *C. cylindrosporum*, and contrasts with the value of 1.0 reported for *C. acidi-urici* by Karlsson and Barker (4). The carbon dioxide incorporated into glycine was found exclusively in the carboxyl group, in agreement with results already reported (19).

TABLE I

Fermentation Products of Uric Acid and Guanine by C. cylindrosporum and C. acidi-urici

Experiment No.	C^{14} source	CO₂ Initial	CO₂ Final	CO₂ Δ	NH₃ Δ	HCOOH Δ	CH₃COOH Δ	CH₂NH₂COOH Initial	CH₂NH₂COOH Final	CH₂NH₂COOH Δ	Recovery C	Recovery N	Oxidation-reduction index
1	Carbon dioxide	131.5	163.0	31.5	50.6	9.3	4.4	0.1	3.4	3.3	98.8	97.4	1.09
2	Formic acid	92.7	116.5	23.8	43.3	11.4	2.4	0.1	4.4	4.3	96.4	96.2	1.03
3	Acetate-1-C^{14}	93.4	114.3	20.9	42.1	15.6	−2.6	0.1	4.4	4.3	82.0	86.0	0.92
4	Acetate-2-C^{14}	90.6	114.6	24.0	44.5	12.1	0.0	0.1	4.6	4.5	82.0	90.7	0.97
5	Glycine-1-C^{14}	113.0	136.0	23.0	46.5	10.3	3.7	18.7	21.2	2.5	83.2	91.0	0.88
6	Glycine-2-C^{14}	91.8	118.0	26.2	48.2	10.2	4.3	19.5	21.4	1.9	92.2	96.5	1.00
7	Guanine-2-C^{14}	98.5	130.0	31.5	49.1	11.2	4.1	0.1	2.9	2.8	103	96.4	1.15
8	Guanine-4-C^{14}	101.2	130.3	29.1	51.5	10.0	4.5	2.0	4.4	2.4	96.2	100	1.05
9	Guanine-6-C^{14}	97.6	121.5	23.9	41.9	8.5	2.8	0.1	4.8	4.7	86.0	86.0	1.04
10	Guanine-8-C^{14}	101.0	127.0	26.0	46.0	10.5	3.4	0.1	3.3	3.2	90.4	91.1	0.98
11	Uric acid-5-C^{14}	129.0	156.5	27.5	35.6	4.8	3.7	0.1	1.5	1.4	106	92.0	0.88
12*	Guanine-4-C^{14}	86.3	123.5	37.2	53.1	1.8	10.1	47.8	44.7	−3.1	97.0	93.0	1.09
13*	Guanine-6-C^{14}	81.0	119.0	38.0	56.2	1.7	11.5	44.4	40.8	−3.6	101	98.0	1.10

The fermentation media contained, per ml., 10 μmoles of guanine, 1 μmole of uric acid (see foot-note 1), the indicated amount of carbonate, salts, yeast extract, and indicators as described under "Methods." Additional supplements were added to give the following initial concentrations per ml.: Experiment 2, 47.9 μmoles of formate; Experiments 3 and 4, 50.9 and 50.1 μmoles, respectively, of acetate. Glycine was added in Experiments 5, 6, 12, and 13 to give the initial concentrations indicated. The initial concentration of ammonia, formate, and acetate was also determined in each experiment and was found to be negligible except when intentionally added as noted. The only substrate added in Experiment 11 was 10 μmoles of uric acid.

* *C. acidi-urici* was used in these experiments; in all others, *C. cylindrosporum*.

The utilization of formic acid was determined in Experiment 2 (Table II). Although 4.4 times the amount of formic acid formed was added at the beginning to the fermentation, the specific activity of the final formic acid recovered was only 0.48 times that originally present. The carbon dioxide was highly labeled, probably through the action of the formic dehydrogenase. The methyl carbon of acetic acid was also labeled and had 9.2 times the activity of the carboxyl carbon. Only small amounts of C^{14} appeared in glycine.

TABLE II

Specific Activity and C14 Recovery in Products of Guanine-C14 and Uric Acid-C14 Fermentations by C. cylindrosporum and C. acidi-urici

Experiment No.	C14 source	Initial	Specific activity, c.p.m. per μmole							Per cent C14 recovered							
						Acetic acid		Glycine		Products			Acetic acid		Glycine		
			CO_2	HCOOH		-COOH	CH_3-	-COOH	CH_3-	CO_2	HCOOH	-COOH	CH_3	-COOH	CH_3	Total
1	Carbon dioxide	35.1	30.0	18.6	1.85	9.7	13.0	(2.0)	106	4.2	0.2	0.9	0.9	0.0	112
2	Formic acid	129	19.6	61.5	2.3	21.2	5.4	1.8	37.2	59.0	0.1	0.8	0.4	0.1	98
3	Acetate-1-C14	1415*	0.15	0.29	1489	7.2	1.55	0.0	0.0	0.0	99.4	0.5	0.0	0.0	100
4	Acetate-2-C14	45.4†	0.0	0.0	0.14	49.6	0.0	0.0	0.0	0.0	0.0	109	0.0	0.0	109
5	Glycine-1-C14	148	1.93	1.49	1.06	0.79	85.4	0.0	9.5	0.5	3.3	1.2	65.4	0.0	75
6	Glycine-2-C14	144	0.38	1.55	21.4	8.0	0.93	103	1.6	0.6	0.3	1.6	0.7	79.0	86
7	Guanine-2-C14	231	17.2	27.6	2.2	0.7	29.7	4.4	87.8	12.2	0.3	1.6	3.4	0.5	106
8	Guanine-4-C14	86.5	5.85	4.67	0.83	2.9	32.2	1.7	80.2	5.0	0.4	1.4	8.1	0.4	95
9	Guanine-6-C14	138	0.88	4.89	0.37	1.6	5.24	0.0	79.0	2.8	0.1	0.3	1.6	0.0	81
10	Guanine-8-C14	82.6	4.31	22.9	2.57	13.5	1.72	1.4	66.2	29.1	1.1	5.6	0.7	0.6	103
11	Uric acid-5-C14	79.4	1.88	3.80	53.7	24.5	2.7	41.5	37.0	2.3	25.0	11.5	0.5	7.8	81
12‡	Guanine-4-C14	80.2	6.0	6.49	2.30	2.17	2.65	0.14	83.9	1.4	2.6	2.5	13.4	0.7	105
13‡	Guanine-6-C14	138	9.51	7.80	0.66	4.12	1.04	0.0	74.3	0.9	0.5	3.1	2.8	0.0	82

* The activity found in the C-2 of the initial acetic acid was 7.9 c.p.m. per μmole.

† The activity found for the C-1 of the initial acetic acid was 1 to 3 per cent of that of C-2 and presumably resulted from randomization during the pyrolysis (14).

‡ C. acidi-urici was used in these experiments; in all others C. cylindrosporum.

Acetic acid was not metabolized significantly, as shown in Experiments 3 and 4.

Experiments 5 and 6 were carried out in the presence of glycine-C^{14}. The recovery of C^{14} was low in both these fermentations, particularly with carboxyl-labeled glycine. Considerable amounts of C^{14} were found in the carbon dioxide formed in the fermentation of guanine in the presence of glycine-1-C^{14} (Experiment 5). The amounts of C^{14} found in formate or acetate were relatively small. In contrast to the carboxyl of glycine, the methylene carbon of glycine was converted to acetic acid to a significant extent (Experiment 6). The ratio of activity of the carboxyl to that of the methyl group was 3, the same value observed in a similar experiment with *C. acidi-urici* (4). The activity found in carbon dioxide and formate in Experiment 6 was small, but the fact that formic acid has a higher specific

TABLE III

Fermentation of Guanine-8-C^{14}

Experiment No.	Time, hrs.	Per cent guanine fermented	Specific activity, c.p.m. per μmole		Ratio of activities, HCOOH:CO$_2$
			CO$_2$	HCOOH	
14*	27.5	59	11.7	80.2	6.85
14*	31.5	74	14.0	92.3	6.60
10†	180	100	4.3	22.9	5.32

* Guanine-8-C^{14}, 363 c.p.m. per μmole.
† Guanine-8-C^{14}, 82.6 c.p.m. per μmole.

activity than carbon dioxide indicates that the methylene group of glycine is converted to formic acid without going through carbon dioxide.

Fermentation of C^{14}-Purines—Experiments 7 through 11 (Table II) report the results obtained in the fermentation of the various C^{14}-purines by *C. cylindrosporum*. It was found that most of the C^{14} of each of the labeled substrates fermented was recovered as carbon dioxide. However, examination of the specific activity of the different carbon atoms of the products discloses the varied fates of the purine carbon atoms.

The products with the highest specific activity formed from C-2 of guanine (Experiment 7) were carbon dioxide, formic acid, and the carboxyl carbon of glycine. The final specific activity of the last two products is greater than that of carbon dioxide and indicates that formic acid and the carboxyl carbon of glycine are not formed via carbon dioxide. The acetic acid formed is labeled mainly in the methyl carbon, and its labeling is similar to that observed in Experiment 1 with $C^{14}O_2$.

Starting with guanine-4-C^{14}, the carboxyl of glycine has the highest

specific activity, as shown by Experiment 8. Carbon dioxide, formic acid, and the methyl of acetic acid were also labeled.

The product with the highest specific activity formed in the fermentation of guanine-6-C^{14} was carbon dioxide (Experiment 9). Only small amounts appeared in the other products, but the specific activities of the carboxyl of glycine and of formic acid were about half of that of the carbon dioxide.

Formic acid and the methyl group of acetic acid had the highest specific activity of the products formed from guanine-8-C^{14} (Experiment 10).

C-5 of uric acid (Experiment 11) gave rise to doubly labeled acetic acid and methylene-labeled glycine of high specific activity. The ratio of C^{14} in the methylene to that in the carboxyl of acetic acid was 0.45, compared to a value of 0.39 reported by Karlsson and Barker (4) for this value in a similar experiment with *C. acidi-urici*. Less of C-5 was converted to carbon dioxide than any other purine carbon atom.

The results obtained in the fermentation of guanine-4-C^{14} and guanine-6-C^{14} by *C. acidi-urici* are given in Experiments 12 and 13. By analogy with the results obtained with *C. cylindrosporum* in Experiment 8, position 4 was expected to form the carboxyl of glycine. Although the specific activity of the glycine isolated was low, owing to its dilution by a pool of glycine added to trap the glycine formed, 13.4 per cent of guanine-4-C^{14} was converted to the carboxyl of glycine, a value somewhat higher than that observed in the fermentation of guanine-4-C^{14} by *C. cylindrosporum*. The results obtained in the fermentation of guanine-6-C^{14} by *C. acidi-urici* (Experiment 13) are very similar to those obtained with *C. cylindrosporum* (Experiment 9) and indicate that position 6 is converted mainly to carbon dioxide by both organisms.

Xanthine-C^{14} Degradation by Cell Extracts—Determination of the sources of carbon dioxide and formic acid in these fermentations, particularly in relation to C-2 and C-8 of purine, was complicated because of the presence of a formic dehydrogenase in the cells, which interconverts these two products (9). A cell extract has been prepared as described under "Methods" which degrades xanthine and is free of formic dehydrogenase (9). When a sample of xanthine-2-C^{14} with an activity of 481 c.p.m. per μmole was degraded by the extract, the specific activity of the carbon dioxide formed was 0.41 c.p.m. per μmole, while that of the formic acid formed was 324 c.p.m. per μmole. With xanthine-8-C^{14} having an initial activity of 880 c.p.m. per μmole, the carbon dioxide formed has an activity of 590 c.p.m. per μmole, while the activity of the formic acid was only 4.0 c.p.m. per μmole. The results show that C-8 is converted to formic acid and that C-2 is converted to carbon dioxide by this preparation.

Fermentation of Uric Acid-7-N^{15}—The fate of N-7 of the purine was

determined in an experiment in which uric acid-7-N^{15} was fermented by *C. cylindrosporum* (Table IV). The fermentation was carried out in the presence of a pool of unlabeled ammonia. Only 0.14 μmole of glycine was formed per micromole of uric acid fermented, but its isotope concentration was equal to that of the N-7 of the fermented uric acid, demonstrating that the N of glycine is derived entirely from the N-7 position of uric acid. The N^{15} found in the ammonia is due to conversion of part of the N-7 of uric acid to this product rather than to non-specific labeling in the uric acid used.

TABLE IV

Glycine Formation from Uric Acid-7-N^{15}

Compound	Concentration		N^{15}
	Initial	Final	
	μmoles per ml.	*μmoles per ml.*	*atom per cent excess*
Uric acid N-7...................	10.0	<0.2	0.37*
" " N(1+3+9).............			0.033*
Ammonia.......................	41.2	79.0	0.052
Glycine.......................	0.0	1.39	0.39

* These values were calculated from the experimental value of 0.12 atom per cent excess of N^{15} found for the uric acid. The calculations are based on the results reported by Shemin and Rittenberg (20).

DISCUSSION

In the previous experiment with *C. acidi-urici*, Karlsson and Barker (4) were able to calculate the per cent of each product carbon atom arising from specific purine carbon atoms of the fermented substrate, from carbon dioxide, and from glycine. The calculations were complicated by the use of biosynthetic samples of purines which were not specifically labeled. This difficulty has been overcome in the experiments reported here through the use of synthetic substrates which were specifically labeled with C^{14} in a single carbon atom. The fermentations have been carried out under essentially the same experimental conditions, and the extent of redistribution of C^{14} among the fermentation products was determined. It is therefore possible to calculate the sources of the carbon atoms of the products formed in the purine fermentation carried out by *C. cylindrosporum*.

F, the per cent of a product derived from a particular substrate atom, can be determined from the specific activity of the designated substrate

atom, s, and the specific activity of the product carbon atom, p, by the following relation,

$$F = (p/s) \times 100$$

However, p must be corrected for C^{14} entering the product from other sources and for dilution of the labeled product by unlabeled material added to the medium. If the specific activity of a second source, s', is higher than p and the per cent contribution from the second source, q, is known, then F may be calculated from the following equation,

$$F = \frac{p - qs'}{s} \times d \times 100$$

where d is the dilution factor or the ratio of final concentration to the concentration that would have been present if none of the compound had been added. This factor is only of significance in calculating the sources of carbon dioxide, when a relatively large pool of this compound was added to the fermentation medium.

Examples of the calculations used have been provided by Karlsson and Barker (4). Although it is not necessary to make corrections for the contribution due to non-specific labeling of the purine molecule in the experiments reported here, except in the fermentation of uric acid-5-C^{14}, it is still necessary to assume a value for the specific activity of secondary products, s', in order to calculate the contribution from such sources. This value has been estimated as the average value, $(s_0 + s_f)/2$, where s_0 and s_f are the initial and final specific activities of a product in a particular fermentation. Such corrections are, fortunately, usually of minor importance. However, the conversion of carbon dioxide to formate, C-2 of acetic acid, and C-1 of glycine, or the contributions of formate to carbon dioxide and C-2 of acetic acid occur to a significant extent, and the calculated values obtained in these cases are less accurate.

The results obtained for the source of each atom of the products, by the method described for the calculations, are shown in Table V. Each vertical column should add up to 100 per cent. The deviation from this value is quite large with formate, but is within 20 per cent of this value with other products. The chief sources of carbon dioxide are C-2, C-4, and C-6 of the purine molecule. Formic acid is derived chiefly from C-8 and smaller amounts from carbon dioxide. The methyl of acetic acid is derived chiefly from C-5, with significant contributions from carbon dioxide and formic acid. The carboxyl group of acetic acid is derived mainly from C-5, although significant amounts also arise from the methylene carbon of glycine. The methylene of glycine arises almost entirely from C-5, while

the carboxyl of glycine is derived from C-4, with an unexpectedly large contribution from carbon dioxide.

The degradation of the purine molecule by *C. cylindrosporum* bears a striking similarity to the general scheme of purine biosynthesis (21). Thus, carbons 1 and 2 and nitrogen of glycine are converted to purine atoms 4, 5, and 7, respectively, and are formed from these same purine atoms in the fermentation. C-8 arises from formate in the biosynthesis and yields formate in the fermentation, while C-6 arises from carbon dioxide

TABLE V

Source of Fermentation Products

Source	Per cent of product carbon atoms derived from indicated source					
	CO_2	HCOOH	Acetic acid		Glycine	
			—COOH	CH₃—	—COOH	—CH₂—
Carbon dioxide..........		28.6	5.5	24.3	39.6	0.0
Formic acid..............	10.2		1.9	19.7	1.6	1.9
Acetate, —COOH........	0.0	0.0		0.0	0.0	0.0
" CH₃—.........	0.0	0.0	0.0		0.0	0.0
Glycine, —COOH........	1.6	1.3	0.9	0.3		0.0
" —CH₂—........	0.2	1.2	17.3	6.5	0.7	
Purine, C-2.............	25.8	≧12.0	0.0	2.1	12.9	1.8
" C-4........	27.2	3.1	0.2	2.0	37.3	2.0
" C-5..........	7.4	0.0	83.1	36.9	2.2	74.0
" C-6.......... .. .	35.3	1.5	0.0	0.0	2.3	0.0
" C-8......	−6 to +11.6	28–91	2.5	10.2	0.6	1.2
Total.......	108–119	76–139	111	102	97	81

in the biosynthesis and yields carbon dioxide in the fermentation. C-2, which is formed from formate in the biosynthesis of purines, is apparently converted to carbon dioxide in the fermentation.

Results obtained with dried cell preparations suggest that these initial products formed in purine fermentation are further metabolized to yield carbon dioxide and acetic acid (22), as has also been suggested by Utter and Wood (23). The labeling of acetate observed in the experiments reported here is consistent with a mechanism in which a C_1 fragment derived from the purine C-5 or C-8, carbon dioxide, or formate condenses with glycine derived from the purine atoms 4, 5, and 7 to form serine, which is converted to pyruvate and finally to acetate (22). In this process, C-4 is lost as carbon dioxide and the methyl of the acetic acid formed is

derived from the various C_1 sources mentioned, while the carboxyl of the acetic acid is derived from purine C-5 or the methylene of glycine. Further evidence in support of such a mechanism is provided by the observations of Radin and Barker (22) in which dried cell preparations of *C. acidi-urici* were shown to catalyze (1) the conversion of glycine to acetate, ammonia, and carbon dioxide, (2) the oxidative deamination of serine to pyruvate, and (3) the oxidation of pyruvate to acetate and carbon dioxide. Although the mechanism involved in the conversion of purine C-5 to the methyl carbon of acetic acid is not established by the fermentation experiments, the results do provide evidence that carbon dioxide and formic acid are not intermediates in the reaction. This is particularly evident from the results of Experiment 11 (Table II), in which the specific activity of the methyl of the acetic acid derived from uric acid-5-C^{14} is much greater than the specific activity of either the carbon dioxide or formate. The results of these fermentations also demonstrate that the purine C-5 is a much better source of the methyl of acetate that the methylene of glycine. The mechanism involved in the extensive incorporation of carbon dioxide into the carboxyl of glycine, which has been observed previously (19), still requires adequate explanation.

The experiments with the cell-free preparation provide evidence that purine C-2 is converted exclusively to carbon dioxide; however, in the fermentation experiment with guanine-2-C^{14}, the formic acid and glycine carboxyl carbon atoms had a higher specific activity than the carbon dioxide, indicating that these atoms were not derived from carbon dioxide. These results suggest either that there is more than one mechanism of purine C-2 metabolism, or that the equilibration between the carbon dioxide produced in the organism and that in the medium is slow, and hence the specific activity of the formate and glycine carboxyl atoms reflects the specific activity of the carbon dioxide within the cell.

The fermentations with *C. acidi-urici* also indicate that C-6 of guanine is converted to carbon dioxide and that the carboxyl of glycine is formed from the C-4 of purine, and would suggest that the chief difference between the purine fermentations carried out by this organism and by *C. cylindrosporum* is in the metabolism of the glycine and formate formed.

SUMMARY

The fermentation of purines by *Clostridium cylindrosporum* and *Clostridium acidi-urici* has been investigated by using C^{14}-labeled guanine, uric acid, glycine, formate, acetate, and carbon dioxide and uric acid-7-N^{15}. The results show that the carbon dioxide is derived mainly from C-2, C-4, and C-6 of the purine molecule. Formic acid is derived chiefly from C-8 and smaller amounts from carbon dioxide. The methyl of acetic

acid is derived chiefly from C-5, with significant contributions from carbon dioxide and formic acid. The carboxyl group of acetic acid is derived mainly from C-5, although significant amounts also arise from the methylene carbon of glycine. The methylene of glycine arises almost entirely from C-5, while the carboxyl of glycine is derived from C-4, with an unexpectedly large contribution from carbon dioxide. The amino group of glycine is derived from N-7.

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PURINE FERMENTATION BY CLOSTRIDIUM CYLINDROSPORUM

II. PURINE TRANSFORMATIONS*

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Two organisms, *Clostridium acidi-urici* and *Clostridium cylindrosporum* which grow on certain purines as the sole carbon and nitrogen source, have been isolated and described by Barker and Beck (1). Uric acid, guanine, and xanthine are readily decomposed by growing cultures of these organisms, whereas hypoxanthine is attacked less readily, and purine, adenine, adenosine, and guanosine are not appreciably decomposed (2). Radin and Barker (3) prepared cell-free extracts of *C. acidi-urici* which actively degrade xanthine and identified glycine, formic acid, carbon dioxide, and ammonia as products. Cell-free preparations of *C. cylindrosporum* have now been prepared by the extraction of lyophilized cells with buffers under anaerobic conditions. The action of this preparation on a variety of purines, many of which were not available in previous studies, has been investigated. The occurrence of guanase, nucleoside phosphorylase, and a xanthine dehydrogenase has been demonstrated in these extracts of *C. cylindrosporum*, and the presence of these enzymes affords some explanation of the substrate specificity observed with the extracts and with growing cultures.

EXPERIMENTAL

Methods—Stock cultures of *C. cylindrosporum*, strain HC1 (1), were grown as described previously (4). For the preparation of washed cell suspensions and cell extracts, the organism was grown in 20 liter glass carboys containing the stock culture medium previously described (4), without sodium thioglycolate or agar. The autoclaved medium was cooled to 37°, and solid sodium hydrosulfite was cautiously added until the green

color of the medium, caused by the methylene blue, disappeared. The contents of the carboy were mixed with a stream of non-sterile nitrogen during this process. Each carboy was inoculated with 2 liters of an 18 hour culture of the organism. Substrate disappearance was determined at hourly intervals, once turbidity was evident, by measuring the optical density (1 cm. cell) at 290 mμ of a 1:100 dilution of the medium. When this value was less than 0.025, the cells were harvested with a Sharples centrifuge and washed with cold distilled water. Three types of preparations were obtained from these cells by the following procedures.

Washed Cells—The cells from a 20 liter culture were suspended in 50 ml. of previously boiled and cooled water, and 2 ml. of this suspension were further diluted to 10 ml. and stored in an evacuated Thunberg tube at 3°.

Cell Extract—The washed cells were lyophilized and stored in an evacuated desiccator at −10°. 500 mg. of the lyophilized cells were incubated with 20 ml. of 0.1 M potassium phosphate or triethanolamine hydrochloride (TEA) buffer at pH 7.0, 0.01 M with respect to cysteine,[1] in an evacuated vessel at 37° for 1 hour. The incubation mixture was centrifuged at 20,000 × g for 10 minutes at 0°, and a clear, enzymatically active extract was obtained. The extract was stored in an evacuated tube at −10°.

Precipitated Extract—The cell extract was saturated with ammonium sulfate, and the precipitated protein was dissolved in oxygen-free 0.1 M potassium phosphate or TEA buffer at pH 7.0, 0.01 M with respect to cysteine.[1] The solution was stored in an evacuated tube at −10°.

Carbon dioxide, ammonia, formic acid, acetic acid, and glycine were determined as previously described (4).[2] Orthophosphate was determined by the method of Lowry and Lopez (5). Ribose-1-phosphate was estimated by the procedure described by Abrams and Klenow (6). Samples were chromatographed by the ascending technique on Whatman No. 1 paper with 5 per cent disodium phosphate-isoamyl alcohol, as described by Carter (7). Uric acid was identified by its spectrophotometric behavior on treatment with a commercial uricase preparation under conditions described by Buchanan *et al.* (8). Spectra were determined in 1 cm. cells with the Beckman model DU spectrophotometer.

Materials—Xanthine, xanthosine, guanine, guanosine, hypoxanthine,

[1] Cysteine was omitted from the buffer in preparations used for studying methylene blue reduction rates.

[2] It should be pointed out that test solutions were heated to 100° at pH 5 in the determination of glycine, at pH 9.5 for the determination of ammonia, and in strong alkali, followed by acid at pH 2, in the determination of the volatile acids. The values obtained for these substances may, therefore, represent certain *bound* forms.

inosine, adenine, and uric acid were obtained from the Nutritional Bio-chemicals Corporation and were used without further purification.

Results

Purine Degradation by Growing Cultures—The ability of *C. cylindro-sporum* to grow when supplied with various purines as the sole carbon and nitrogen source was determined (Fig. 1). Growth was estimated by measuring the amount of ammonia formed (1). Xanthine supported the most rapid growth of the organism, although the absolute amount of ammonia formed from uric acid was somewhat greater.
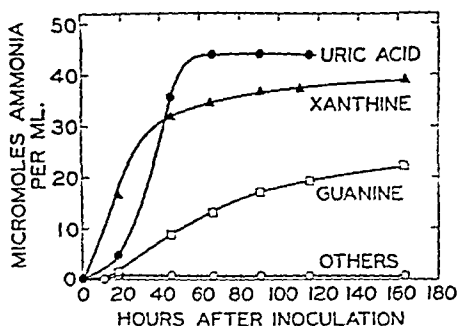
FIG. 1. Growth of *C. cylindrosporum* on various purines. The cultures were grown in 25 ml. volumetric flasks containing 10 μmoles per ml. of the purine indicated and the usual growth medium (4). Oxsorbent was used to remove oxygen from the sealed flasks. Aliquots of the medium were removed aseptically at the times indicated for ammonia analysis and the flasks were resealed. The purines tested in the curve marked "others" were hypoxanthine and xanthosine.

In similar experiments, previously reported by Barker and Beck (2) using *C. acidi-urici*, uric acid was found to be utilized much more rapidly than xanthine. The apparent discrepancy between these two results is probably explained by the use of an impure sample of xanthine in the earlier study.

Purine Degradation by Washed Cells—The degradation of a variety of purines by cell suspensions of *C. cylindrosporum* was investigated with washed cells grown on uric acid. Activity was determined by measuring carbon dioxide formation in the Warburg apparatus (Table I). Of the compounds tested, xanthine was most rapidly degraded. The other active compounds found were guanine, uric acid, 6,8-dihydroxypurine, and guanosine.

Products Formed from Xanthine by Cell Extracts—A soluble extract of *C. cylindrosporum*, prepared as described, was found to convert xanthine

to products without significant absorption above 230 mμ (Fig. 2). The products of the reaction are shown in Table II. However, the methods used in the determination of ammonia, formic acid, and glycine involved

TABLE I

Decomposition of Purines by Washed Cell Suspensions of C. cylindrosporum

Compound	Substituents on purine ring				Relative activity*
	2-	6-	8-	9-	
Hypoxanthine..................		—OH			–
Inosine......................		—OH		Ribosyl	–
†			—OH		–
Xanthine....................	—OH	—OH			100
Xanthosine..................	—OH	—OH		Ribosyl	–
†	—OH	—OH	—CH₃		–
†		—OH	—OH		7.8
Uric acid...................	—OH	—OH	—OH		4.4
‡	—OH	—OH	—OH	Ribosyl	–
Adenine....................		—NH₂			–
Guanine....................	—NH₂	—OH			<3.9
Guanosine..................	—NH₂	—OH		Ribosyl	18
Isoguanine†..................	—OH	—NH₂			–
†		—NH₂	—OH		–
†	—NH₂	—OH	—OH		–
†	—OH	—NH₂	—OH		–
†	—NH₂	—NH₂	—OH		–

The side arm of the Warburg flask contained 0.5 ml. of the washed cell suspension. The main compartment contained 1 ml. of 0.01 M substrate in 0.1 M potassium phosphate, pH 7.0, 0.2 ml. of neutralized 0.3 M cysteine hydrochloride in 0.5 M potassium phosphate, pH 7.0, and 0.05 M potassium phosphate, pH 7.0, to make the total volume 2.2 ml. The flasks were flushed with hydrogen for 3 minutes and equilibrated at 36° for 5 minutes before tipping in the enzyme. The manometers were read at 5 minute intervals until the carbon dioxide evolution had stopped.

* The time required for the formation of half the final amount of CO_2 evolved was determined. This value was 7 minutes for xanthine. The activity is expressed as the reciprocal of this value relative to xanthine, which is set at 100. The dash indicates that no demonstrable degradation was observed.

† Gifts of Dr. H. O. L. Fischer from the collection of E. Fischer.

‡ A gift of Dr. C. E. Carter.

distillation at 100° from alkaline or acid media, and these substances may have been formed by the decomposition of unstable enzymatic products during the analytical procedures used.

Nucleoside Phosphorylase—A preparation free of measurable phosphate was obtained by precipitating the proteins of the cell extract with saturated ammonium sulfate as described. Xanthine was readily degraded by this

preparation (Fig. 3), and the addition of orthophosphate had no effect. Guanosine was not degraded unless orthophosphate was added, and the
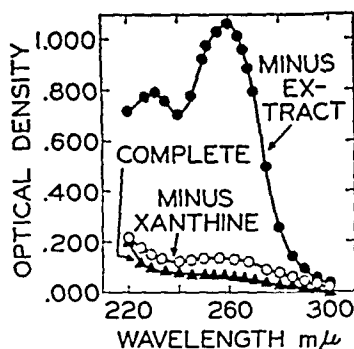

FIG. 2. Xanthine degradation by extracts of *C. cylindrosporum*. The complete system consisted of 20 μmoles of xanthine, 50 μmoles of cysteine, 500 μmoles of potassium phosphate, pH 7.0, 5 μmoles of ferrous sulfate, 0.5 ml. of cell extract, and water to bring the volume to 4.0 ml. The stoppered tubes were incubated at 37° for 1 hour. The reaction was stopped by the addition of 1 ml. of 15 per cent perchloric acid to each tube. The spectrum of the complete system is corrected for the absorption due to the extract.

TABLE II

Products Formed from Xanthine by Extracts of C. cylindrosporum

	Initial	Final	Δ	Moles per mole xanthine utilized
	μmoles per ml.	*μmoles per ml.*	*μmoles per ml.*	
Xanthine	10.0	2.4	−7.6	
Carbon dioxide	2.4	14.8	12.4	1.6
Ammonia	5.7	23.8	18.1	2.4
Formic acid	1.6	9.0	7.4	0.97
Glycine	0.0	5.4	5.4	0.71

The main compartment of the Warburg vessel contained 100 μmoles of potassium phosphate, pH 7.0, and 0.3 ml. of an extract of *C. cylindrosporum*. After equilibration under a hydrogen atmosphere, 10 μmoles of xanthine were added from a side arm. Sulfuric acid was added from another side arm after 30 minutes at 37°. Initial values were obtained from a duplicate flask incubated without added xanthine.

products formed had no absorption in the range of 230 to 300 mμ. Guanosine disappearance was accompanied by the disappearance of an equivalent amount of orthophosphate, but without change in the pentose concentration. The rate of hydrolysis of the phosphate ester is similar to that for ribose-1-phosphate (Fig. 4) (6). Inosine as well as guanosine formed easily hydrolyzable phosphate; no reaction was detected with

adenosine or xanthosine.   These observations suggest the occurrence of a nucleoside phosphorylase in *C. cylindrosporum* having a specificity similar to that of the mammalian enzyme (Kalckar (9)).

Guanase—The occurrence of guanase in the cell extract could be demonstrated, since, when guanosine is incubated with the precipitated extract in the presence of oxygen, the original spectrum is replaced by one almost identical with that of xanthine (Fig. 5).   The xanthine formed, calculated
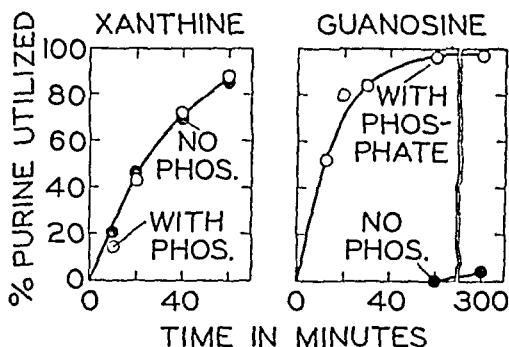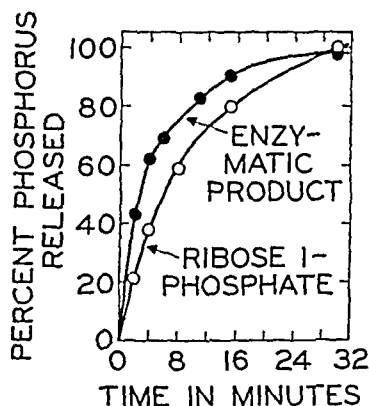

FIG. 3

FIG. 4

FIG. 3. Effect of orthophosphate on purine degradation by purified extracts. Stoppered tubes containing 10 μmoles of purine, 200 μmoles of potassium phosphate, pH 7.0, where indicated, and water to make volume to 10 ml. were incubated at 37°. The reaction was stopped at the times indicated by adding a 1 ml. aliquot to 9 ml. of 6 per cent perchloric acid.   The purine content of the sample was determined from the absorption of the filtrate at 255 mμ, corrected for absorption due to the enzyme blank.

FIG. 4. Rate of release of orthophosphate from the enzymatic reaction product and ribose-1-phosphate.   Stoppered tubes containing 20 μmoles of guanosine, 80 μmoles of cysteine, 20 μmoles of potassium phosphate, pH 7.0, and 1.2 ml. of the precipitated enzyme were incubated at 37° for 90 minutes.   Samples of the incubation mixture were deproteinized and hydrolyzed with 1 per cent perchloric acid. The hydrolysis curve for ribose-1-phosphate is taken from the data of Abrams and Klenow (6).

from the absorption at 260 mμ, was equivalent to 93 per cent of the initial guanosine present.   The conversion of guanine to xanthine was demonstrated in a similar experiment, although it was not as complete in this case because of the insolubility of guanine.   The formation of xanthine from each substrate was confirmed by paper chromatography of the reaction mixtures in 5 per cent disodium phosphate-isoamyl alcohol (7).

The formation of xanthine from guanosine requires the action of nucleoside phosphorylase, followed by that of guanase.   Evidence for this sequence of reactions is given in the following section.

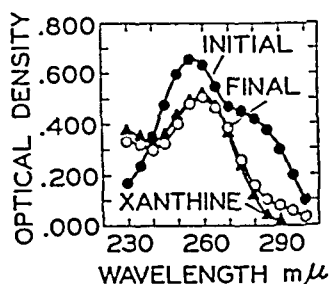*Xanthine Dehydrogenase*—The reduction of methylene blue in the pres-

Fig. 5. Enzymatic conversion of guanosine to xanthine. Tubes containing 2 μmoles of guanosine, 200 μmoles of potassium phosphate, pH 7.0, and 1 ml. of the precipitated enzyme in a volume of 2.5 ml. were incubated at 37° for 2 hours. The reaction was stopped by the addition of perchloric acid to give a final concentration of 5 per cent. Absorption spectra were determined in 5 per cent perchloric acid and have been corrected for the absorption due to the enzyme.

TABLE III

Relative Rates of Methylene Blue Reduction by Suspensions and Extracts of C. cylindrosporum with Various Substrates

Substrate	Rate	
	Suspension	Extract
Xanthine	100	100
Guanine	20	25
Guanosine	12	29
Guaninedeoxyriboside		15
6,8-Dihydroxypurine	20	18
Hypoxanthine	12	4
8-Hydroxypurine	2	1
Adenine......	1	1
Uric acid......	1	1
Formic acid.......	29	1
Glycine	9	1
DL-Serine...	9	1
Pyruvic acid...	7	1
Salicylaldehyde . .		1
" + xanthine .		100
Blank . .	1	1

The Thunberg tube contained 2 μmoles of substrate, 150 μmoles of potassium phosphate, pH 7.0, and 0.6 μmole of methylene blue in a total volume of 2.3 ml. The cap contained 0.2 ml. of the washed cell suspension or 0.5 ml. of the precipitated extract. The evacuated tubes were equilibrated at 35° for 3 minutes and then mixed. The relative reduction rates were calculated from the reciprocals of the time required for complete decolorization, as determined visually. In the presence of xanthine, the time required was less than 2 minutes. The blanks did not reduce the dye in less than 2 hours.

ence of a cell suspension of *C. acidi-urici* and a variety of substrates was observed by Barker and Beck (2). Of the various purines and amino acids tested, methylene blue reduction occurred most rapidly in the
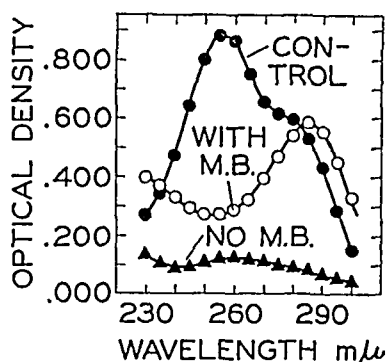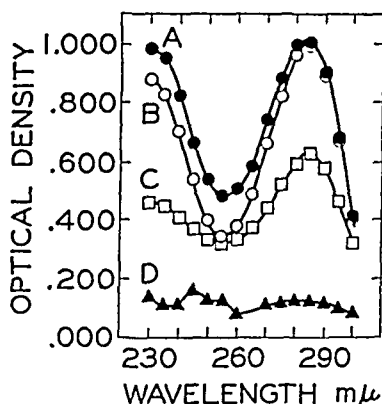

FIG. 6   FIG. 7

FIG. 6. Guanosine decomposition in the presence and absence of methylene blue. Evacuated Thunberg tubes contained 10 μmoles of guanosine, 100 μmoles of potassium phosphate, pH 7.0, 0.5 ml. of the precipitated extract, and 7.5 μmoles of methylene blue, where indicated, in a total volume of 2.5 ml. The tubes were incubated for 1.5 hours, when methylene blue reduction was complete, and were then placed in a boiling water bath for 10 minutes. Guanosine was added to the control tube after heating. The absorption spectra were determined after diluting 0.1 ml. of the boiled supernatant solution with 7.0 ml. of 6 per cent perchloric acid.

FIG. 7. Conversion of 6,8-dihydroxypurine to uric acid. An evacuated Thunberg tube containing 10 μmoles of 6,8-dihydroxypurine, 7.5 μmoles of methylene blue, 0.7 ml. of the precipitated extract, and 100 μmoles of potassium phosphate, pH 7.0, in a total volume of 2.5 ml. was incubated at 35° for 1.5 hours. The reaction was stopped by placing the tubes in a boiling water bath for 10 minutes. Curve A is the absorption curve found for the enzymatically formed component obtained after paper chromatography of 0.1 ml. of the incubation mixture with 5 per cent disodium phosphate-isoamyl alcohol (7) and elution of the spot with 0.1 M potassium phosphate, pH 7.0. Curve B is the absorption spectrum of uric acid. Curve C is the absorption of the supernatant solution at a dilution equal to that used for Curve D. Curve D was obtained by treating 0.5 ml. of the boiled incubation mixture with 50 μmoles of borate buffer, pH 9.2, and 100 mg. of commercial uricase powder in a volume of 7.5 ml. for 2.5 hours at 43°. A correction for the absorption due to uricase has been supplied.

presence of glycine. Among the purines, only xanthine and guanine were observed to cause a rapid rate of methylene blue reduction.

The relative rates of methylene blue reduction with washed cell suspensions and precipitated extracts of *C. cylindrosporum* with various purines is shown in Table III. The most rapid reduction rate was observed on the addition of xanthine.

Examination of the deproteinized filtrates from these reaction mixtures

showed almost complete disappearance of the substrate spectrum in the absence of methylene blue, but in the presence of the dye the spectrum of the purine was replaced by one having a peak at 285 mμ. Similar results were obtained with xanthine, guanine, 6,8-dihydroxypurine, and hypoxanthine. Paper chromatograms of these incubation mixtures in 5 per cent disodium phosphate-isoamyl alcohol (7) showed an ultraviolet-absorbing component having the same mobility as uric acid in addition to variable amounts of unchanged substrate and methylene blue. The absorption spectrum of the enzymatically formed substance was determined after it

TABLE IV

Effect of 2-Amino-4-hydroxy-6-pteridinecarboxaldehyde on Xanthine Degradation

PTCA	Activity	
	Methylene blue reduction time*	50 per cent xanthine decomposition†
γ per ml.	min.	min.
0	13	55
3	>180	
15	>180	55
30	>180	

* Evacuated Thunberg tubes containing 2 μmoles of xanthine, 0.6 μmole of methylene blue, 25 μmoles of potassium phosphate, pH 7.0, PTCA as indicated, 0.3 ml. of the precipitated extract, and water to make the volume to 3.0 ml. were incubated at 35°, and the time for complete dye reduction was determined visually.

† Stoppered tubes containing 4.0 μmoles of xanthine, 64 μmoles of cysteine, 100 μmoles of TEA, pH 7, 100 μmoles of potassium phosphate, pH 7, 0.4 ml. of precipitated extract, and water to make the volume to 4.0 ml. were incubated at 35°. Samples, removed at various time intervals, were added to 3 per cent perchloric acid, and xanthine disappearance was determined from measurement of the optical density at 260 mμ. Activity is expressed as the time required for 50 per cent degradation.

had been eluted from the paper and was found to agree with that of uric acid (Fig. 6). Further evidence confirming the identity of the product of these reactions as uric acid was obtained by demonstrating the susceptibility of the product to degradation by uricase (Fig. 7). It has not been possible to demonstrate the quantitative conversion of any of the purines to uric acid by the crude or purified extract under the conditions employed. It was found that methylene blue reduction in the presence of guanosine and the precipitated enzyme is dependent on the addition of orthophosphate. No such orthophosphate requirement was observed for the reduction of methylene blue in the presence of xanthine and the precipitated enzyme.

2-Amino-4-hydroxy-6-pteridinecarboxaldehyde (PTCA) has been shown to inhibit xanthine dehydrogenase (10). This compound was also found to

inhibit completely the xanthine dehydrogenase activity of *C. cylindrosporum* extracts (Table IV).   However, PTCA had no demonstrable effect on xanthine degradation under anaerobic conditions in the absence of methylene blue.

The formation of xanthine from uric acid has not been demonstrated directly with the crude or purified extract, since xanthine is further degraded under anaerobic conditions by these preparations.   However, uric acid degradation occurs in the presence of reduced benzyl viologen (Fig. 8).   This
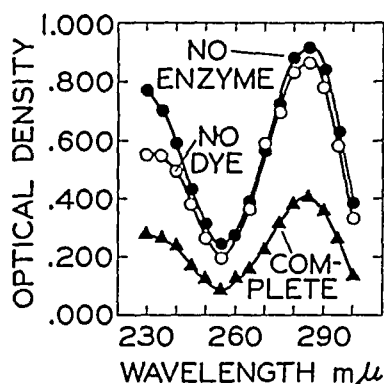
Fig. 8. Effect of benzyl viologen on uric acid utilization.   About 5 mg. of benzyl viologen in 5 ml. of water were reduced with hydrogen in the presence of palladinized asbestos.   The experimental tube contained 2 μmoles of uric acid, 0.5 ml. of the benzyl viologen solution, 0.5 ml. of the precipitated extract, and 100 μmoles of potassium phosphate, pH 7.0, in a total volume of 2.5 ml.   A control tube with no enzyme and one with no dye were also prepared.   The tubes contained some palladium-asbestos and were filled with hydrogen.   They were incubated at 35° for 30 minutes and then placed in boiling water for 5 minutes.   The absorption spectrum of the heated reaction mixture was determined after diluting 1 ml. with 10 ml. of 6 per cent perchloric acid.   The curves have been corrected, where necessary, for the absorption due to the enzyme or the dye.

experiment therefore provides evidence for the reversal of the xanthine dehydrogenase-catalyzed reaction.

Degradation of Pyrimidine and Imidazole Derivatives—The ability of the cell extract to degrade a variety of pyrimidine and imidazole derivatives under conditions in which xanthine is readily degraded was tested.   The disappearance of compounds was followed by the Bratton-Marshall test (11) when possible or by spectrophotometric measurements.   The following compounds were tested: 4-amino-2,6-dihydroxy-5-formamidopyrimidine,[3] 2,4-dihydroxy-5,6-diaminopyrimidine,[3] 5-aminobarbituric acid,[3] 5-ureido-2,4,6-trihydroxypyrimidine,[4] 2,4-diamino-6-hydroxy-5-formamidopyrimi-

[3] Gift of Dr. G. B. Brown.
[4] Gifts of Dr. H. O. L. Fischer from the collection of E. Fischer.

dine,[3] 4-amino-5-imidazolecarboxamide,[5] 4-hydroxy-5-imidazolecarboxamide,[6] 4-formamido-5-imidazolecarboxamide,[6] 4-guanidinoimidazole,[3] and 4-imidazolecarboxylic acid.[7] None of the compounds tested, other than xanthine, was degraded under the test conditions.

DISCUSSION

Although *C. acidi-urici* and *C. cylindrosporum* were first differentiated on a morphological basis (1), a difference in the fermentation products formed by the two organisms was found after further study (2). *C. cylindrosporum* forms glycine as a product of purine fermentation, while none of this amino acid can be detected in fermentation liquors of *C. acidi-urici* (2); furthermore, *C. cylindrosporum* forms 1.0 mole of formic acid and 0.4 mole of acetic acid per mole of guanine fermented, while *C. acidi-urici* forms 1.0 mole of acetic acid and only 0.2 mole of formic acid (4). However, cell-free preparations of both organisms were found to degrade xanthine to yield ammonia, carbon dioxide, formic acid, and glycine or products which yield these substances during the analytical procedures used. In contrast to growing cultures, no acetic acid is formed from purines by the cell-free preparations which have been used.

The central rôle of xanthine in the metabolism of *C. acidi-urici* has been demonstrated by Beck (12), in adaptation studies, and by Radin and Barker (3), who obtained extracts of the organism capable of degrading xanthine but not uric acid. A similar central rôle of xanthine in the metabolism of *C. cylindrosporum* is indicated in the experiments described here. Thus, of all the purines tested, xanthine is the most readily utilized for growth and is the most rapidly decomposed by washed cell preparations. Uric acid, guanine, guanosine, and 6,8-dihydroxypurine were also active in at least one of these tests, and evidence for the conversion of these compounds to xanthine has been obtained. Hypoxanthine, xanthosine, uric acid riboside, various guanine isomers, and other purines were not actively decomposed. The specificity in the utilization of purines and their derivatives for growth by this organism can be partially explained in the terms
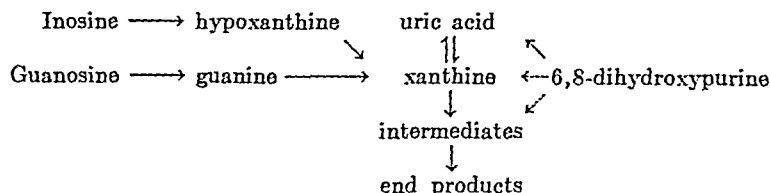
[5] Gift of Dr. S. Gurin.
[6] Gift of Dr. E. Shaw.
[7] Gift of Dr. R. G. Jones.

of the specificity of a number of enzymes shown to be present in the organism. The reactions to be discussed and the central rôle of xanthine are indicated in the accompanying scheme of the purine transformations carried out by *C. cylindrosporum.*

Guanase present in the extract converts guanine to xanthine, but is apparently not active on isoguanine. The available evidence indicates that nucleosides are utilized by this organism only after conversion to the free bases in a reaction catalyzed by nucleoside phosphorylase. Guanosine is thus converted to the free base guanine, which is then rapidly metabolized. Inosine is readily converted to hypoxanthine, presumably by the nucleoside phosphorylase, but the base formed is degraded very slowly. The nucleoside phosphorylase is not active on xanthosine or uric acid riboside (Table I), hence the inability of cell extracts to decompose these two ribosides.

A xanthine dehydrogenase is present in the extracts which oxidizes xanthine to uric acid in the presence of methylene blue. Evidence for the reversibility of this reaction has also been obtained, and it would appear that utilization of uric acid for growth depends on the action of this enzyme with a reducing agent normally present in the cell rather than on the dye used in these experiments. In contrast to the avian xanthine dehydrogenase (10), this enzyme does not oxidize salicylaldehyde and oxidizes hypoxanthine slowly. The inability of hypoxanthine to support growth of the organism can therefore be explained on the basis of the specificity of this enzyme. Uric acid is probably formed from 6,8-dihydroxypurine through the action of xanthine dehydrogenase. Preparations of xanthine oxidase from whey or ox spleen have been shown to reduce dyes in the presence of 6,8-dihydroxypurine, although the presumed product of the reaction, uric acid, was not identified (13). Examples of xanthine oxidase-catalyzed oxidation at C-2 of purines are provided by the action of this enzyme on adenine (14) and hypoxanthine. However, the steps involved in the degradation of 6,8-dihydroxypurine in the absence of methylene blue to products with no ultraviolet absorption are not known. Washed cells decompose 6,8-dihydroxypurine at a higher rate than either uric acid or hypoxanthine. Cell extracts which actively decompose 6,8-dihydroxypurine do not act on either uric acid or hypoxanthine. It therefore seems unlikely that 6,8-dihydroxypurine is converted to a mixture of uric acid and hypoxanthine in a reaction analogous to the anaerobic dismutation of xanthine catalyzed by xanthine oxidase (15). It is possible, however, that 6,8-dihydroxypurine is oxidized to uric acid by xanthine dehydrogenase, and that uric acid is then reduced to xanthine in a coupled reaction. Studies with a purified preparation of xanthine dehydrogenase will be necessary to clarify this reaction.

SUMMARY

Of all the compounds tested, xanthine is most readily utilized for growth of *Clostridium cylindrosporum* and is most rapidly decomposed by washed cell suspensions of the organism. Soluble extracts which degrade xanthine have been prepared from lyophilized cells. Ammonia, carbon dioxide, formic acid, and glycine have been identified as products of the reaction. The methods used for the determination of these products do not eliminate bound forms of these substances as the true enzymatic products. The presence of nucleoside phosphorylase and guanase in the extract has been demonstrated. A xanthine dehydrogenase, which converts xanthine to uric acid in the presence of methylene blue, has also been demonstrated. The action of these enzymes is discussed in relation to the substrate specificity observed with the growing organism and the cell extracts.

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PURINE FERMENTATION BY CLOSTRIDIUM CYLINDROSPORUM

III. 4-AMINO-5-IMIDAZOLECARBOXYLIC ACID AND 4-AMINOIMIDAZOLE*

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Cell-free extracts of *Clostridium acidi-urici* (1) and of *Clostridium cylindrosporum* (2) have been described which degrade xanthine to glycine, formic acid, carbon dioxide, and ammonia or products which yield these substances in the analytical procedures used. The intermediates involved in this process, however, are not known. Evidence exists for two general schemes of purine fermentation. The first of these involves an initial splitting of the imidazole ring of the purine to yield pyrimidine derivatives as intermediates. Whitely (3) has shown the formation and subsequent degradation of uracil and thymine in the fermentation of purines by *Micrococcus aerogenes*. The second scheme involves an initial hydrolytic split of the pyrimidine ring to yield imidazole derivatives as intermediates. Evidence for this scheme of purine degradation was obtained by Radin and Barker (1) using extracts of *C. acidi-urici*. They found that, on incubation of xanthine with extracts of *C. acidi-urici*, substances were formed which reacted in the Pauly test. These compounds were presumed to be imidazole derivatives, but were not identified. In experiments already described (2), a number of available hydrolytic products of xanthine as well as other pyrimidine and imidazole derivatives were tested as possible intermediates in the degradation of xanthine by extracts of *C. cylindrosporum* and were found to be inactive.

Conditions have now been found under which an intermediate in purine degradation is accumulated by extracts of *C. cylindrosporum*. This com-

pound has been identified as 4-amino-5-imidazolecarboxylic[1] acid and has been synthesized. Its enzymatic decarboxylation to 4-aminoimidazole has also been demonstrated.

EXPERIMENTAL

Methods—Strain HC1 of *C. cylindrosporum* was used. Stock cultures (4), cell extracts, and precipitated extracts (2) were prepared as described previously. Whatman No. 1 paper was used for paper chromatography. Samples were applied as 0.01 ml. aliquots and were dried in a current of air at room temperature. The chromatograms were developed by the ascending technique with solvent systems described in the text and were usually run for 15 to 18 hours, when the solvent had traveled about 30 cm. The papers were dried at room temperature. Compounds were detected with the Mineralight lamp (5) and were eluted from the paper with water when necessary. Spectra were determined in 1 cm. cells with the Beckman model DU spectrophotometer. Dowex 50 H+ was prepared just prior to use by passing 5 N hydrochloric acid and water over a column of the resin. Rubber connections were avoided in order to minimize contamination with ultraviolet-absorbing materials. The flow rate was adjusted to 15 to 20 drops per minute. The optical densities of the fractions were determined within 15 minutes after collection, and samples were diluted to give optical density readings of less than 1.0 when necessary. The proper dilution of the eluting salt mixture was used in the reference cell. C^{14} was determined with a thin window Geiger counter. Samples, treated with 1 drop of 5 N potassium hydroxide and a drop of detergent solution, were dried in polyethylene cups at 80°. The variable amounts of salt present in the fractions and the resulting large variability in self-absorption precluded the calculation of C^{14} recovered.

Diazotizable amines were determined, by a slight modification of the procedure described by Bratton and Marshall (6), as follows: The sample, deproteinized when necessary with perchloric acid and diluted with water to a volume of 0.2 ml., was added to 5 ml. of 5 per cent perchloric acid. Then, 0.5 ml. of 0.1 per cent sodium nitrite was added. After 3 minutes, 0.5 ml. of 0.5 per cent ammonium sulfamate was added, followed 2 minutes later by 0.5 ml. of 0.1 per cent *N*-1-naphthylethylenediamine dihydrochloride. After 10 minutes, the optical density at 510 mμ was determined with the Coleman junior spectrophotometer and 16 mm. test-tubes. 1 μmole of 4-aminoimidazole, 4-amino-5-imidazolecarboxylic acid (calculated from the N content of freshly prepared solutions of the compounds), or 4-amino-5-imidazolecarboxamide is equivalent to an optical density reading of 4.5, 4.5, or 4.6, respectively.

[1] Positions 4 and 5 of the imidazole ring are equivalent and may also be referred to as positions 4(5)- or 5(4)-.

The procedure used for analyzing mixtures containing 4-aminoimidazole and 4-amino-5-imidazolecarboxylic acid is based on the selective absorption of 4-aminoimidazole by Dowex 50 NH_4^+ from a solution at pH 8. In practice, 0.5 ml. of a reaction mixture at pH 8.0 was passed over a 2 cm. $\times$ 0.6 sq. cm. column of Dowex 50 NH_4^+. The column was washed with 4.0 ml. of water. The combined effluent and wash waters contained 4-amino-5-imidazolecarboxylic acid, which was then determined by the Bratton-Marshall test. The column was treated with 10 ml. of 5 M formic acid, 1 M with respect to hydrochloric acid, in order to elute the absorbed 4-amino-imidazole. This substance was then determined by the Bratton-Marshall test.

Materials

Xanthine, obtained from the Nutritional Biochemicals Corporation, was used without further purification. Diethanolamine (DEA) and triethanol-amine (TEA) buffers were prepared by neutralization with hydrochloric acid of the free bases, which were obtained from Eastman Organic Chemicals. Samples of xanthine-8-C^{14} and xanthine-2-C^{14} were prepared from the corresponding samples of guanine-C^{14} by treatment with nitrous acid (7) and were purified by chromatography as described by Carter (5).

Solutions of 4-aminoimidazole were prepared by the catalytic reduction of 4-nitroimidazole. The latter compound was obtained by the nitration of imidazole (Eastman Organic Chemicals) (8). The 4-nitroimidazole was recrystallized from glacial acetic acid. $C_3H_3N_3O_2$, calculated, C 31.9, H 2.65, N 37.2 per cent; found, C 31.96, H 2.67, N 37.02 per cent.

The following procedure was used for the reduction: A suspension of 114 mg. of 4-nitroimidazole, 0.8 ml. of 1 M monopotassium phosphate, 14.0 ml. of water, and 100 mg. of 5 per cent palladium-charcoal (Baker and Company, Inc., Newark, New Jersey) was shaken under 1 atmosphere of hydrogen until the gas uptake was complete. The rate of uptake was linear and under the conditions used was complete after 30 to 45 minutes. At that time, 3 moles of hydrogen had been utilized per mole of 4-nitroimidazole. Then 1.2 ml. of 1 M dipotassium phosphate, 0.08 ml. of 2 M sodium sulfide, 0.04 ml. of 4 N hydrochloric acid, and 3.9 ml. of water were added. The solution was filtered with filter aid and stored at 0° in a syringe closed with a sealed hypodermic needle.[2] Samples could thus be withdrawn as needed without exposing the solution to oxygen. The initially colorless solution became yellow after about a day. However, very little change in the di-azotizable amine value of the solution was observed even after storage for several months under these conditions. The stability of a solution of 4-aminoimidazole was determined (Table I). Exposure to oxygen results in the formation of blue pigments and in partial loss of the diazotizable amine.

[2] This method of storage was suggested by Dr. S. Korkes.

Solutions of 4-amino-5-imidazolecarboxylic acid were prepared by the catalytic reduction of 4-nitro-5-imidazolecarboxylic acid. The latter compound was synthesized by the procedure of Allsebrook et al. (9), with a series of reactions described by Fargher and Pyman (8) and by Windaus and Langenbeck (10). 4-Nitro-5-imidazolecarboxylic acid, $C_4H_3O_4N_3$, calculated, C 30.6, H 1.9, N 26.7 per cent; found, C 29.84, H 1.96, N 26.35 per cent.

The procedures for the reduction of the nitro group and for storage of the product are similar to those just described for the preparation of 4-aminoimidazole, except that the reduction was carried out in phosphate buffer at pH 8.5 and the product was stored at this pH. Just before use,

TABLE I

Stability of Solutions of 4-Aminoimidazole

Conditions	Half life at 23°	Half life at 100°
	hrs.	*hrs.*
5% perchloric acid	7	
0.1 N HCl	42	0.083
pH 5 (0.1 M sodium acetate)	>144	0.67
" 7 (0.005 M potassium phosphate)	76	2.3
" 9 (0.1 M DEA)	37	0.13
0.1 N NaOH	5	1.0
5 N NaOH	18	

Samples initially containing 2.5 μmoles of synthetic 4-aminoimidazole per ml· were incubated under the conditions indicated. Aliquots were removed after various time intervals, and diazotizable amine was determined by the Bratton-Marshall test.

the solution was passed over a column of Dowex 50 NH_4^+ to remove 4-aminoimidazole which is formed by spontaneous decarboxylation.

Results

Formation of Intermediate—The rapid formation of a diazotizable amine was observed when xanthine was incubated with an extract of *C. cylindrosporum* (Fig. 1). The disappearance of the amine indicated the possible rôle of the compound (or compounds) formed as an intermediate in purine degradation.

A study was made to determine the pH optimum of the system leading to the formation of the intermediate from xanthine, preliminary to separating this activity from the one responsible for the further degradation of the accumulated amine (Fig. 2). At pH 8.4,[3] the initial rate of formation of

[3] The pH of the incubation mixture at the end of the incubation period was 8.4 as determined with the glass electrode, although a DEA buffer at pH 9.0 was used.

the diazotizable amine was somewhat slower than that observed at pH 6.8, but the amount eventually formed was almost 4 times as great, and no decomposition of the compound was evident.

Paper chromatograms of the reaction mixtures incubated at pH 6.8 or 8.4 were developed with n-propanol-acetic acid (11) and showed the formation
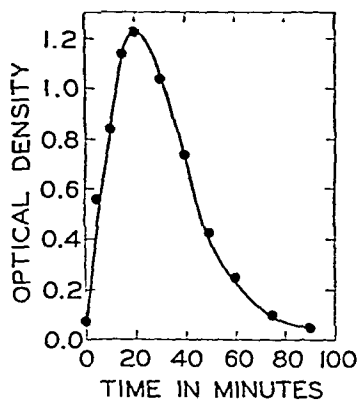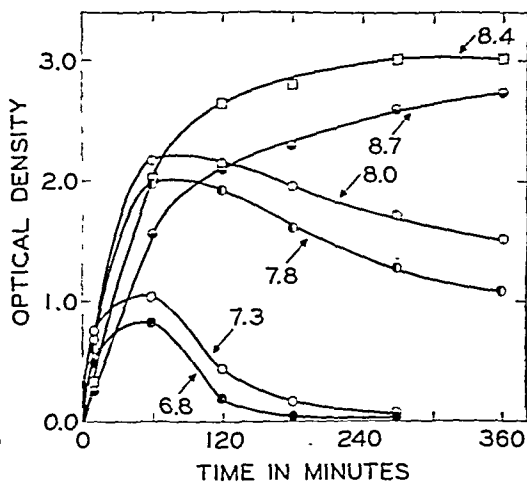

FIG. 1.

FIG. 2.

FIG. 1. Formation of an intermediate in the degradation of xanthine by precipitated extracts of *C. cylindrosporum*. A stoppered tube containing 50 μmoles of xanthine, 200 μmoles of potassium phosphate, pH 7.0, 100 μmoles of TEA, pH 7.0, 180 μmoles of cysteine, 1.0 ml. of the precipitated enzyme, and water to make the volume 10 ml. was incubated at 35°. The reaction was stopped and deproteinized after the time intervals indicated by adding a 0.1 ml. aliquot of the reaction mixture to 5 ml. of 5 per cent perchloric acid. The supernatant solution was assayed by the Bratton-Marshall test.

FIG. 2. Effect of pH on the formation and degradation of an intermediate from xanthine by extracts of *C. cylindrosporum*. Stoppered tubes contained 40 μmoles of xanthine, 20 μmoles of TEA buffer (final pH 6.8, 7.3, 7.8) or DEA buffer (final pH 8.0, 8.4, 8.7), 0.2 ml. of cell extract, 72 μmoles of cysteine, and water to make the volume 4.0 ml. Aliquots of 0.1 ml. were added to 5 ml. of 3 per cent perchloric acid after the time intervals indicated, and diazotizable amine was determined.

of a single component which could be detected as a quenching area when viewed with the Mineralight lamp (5), as a blue area when sprayed with the Pauly reagent for imidazoles (11), and as an orange-pink area when sprayed with the Bratton-Marshall reagents (12). The compound, when eluted from chromatograms, was degraded on reincubation with the cell extract at pH 7.0.

Experiments with Xanthine-C14—The diazotizable amine formed from xanthine was completely adsorbed on Dowex 50 H+, and about 70 per cent

was adsorbed on Dowex 1 chloride.   On chromatographing the incubation mixture of xanthine-8-C[14] or xanthine-2-C[14] with the cell extract (Figs. 3 and 4), six components[4] could be distinguished by ultraviolet absorption. Two of these, Components D and F, were found to give a diazotizable amine test.   Both compounds retained C-8 but not C-2 of xanthine.   To-
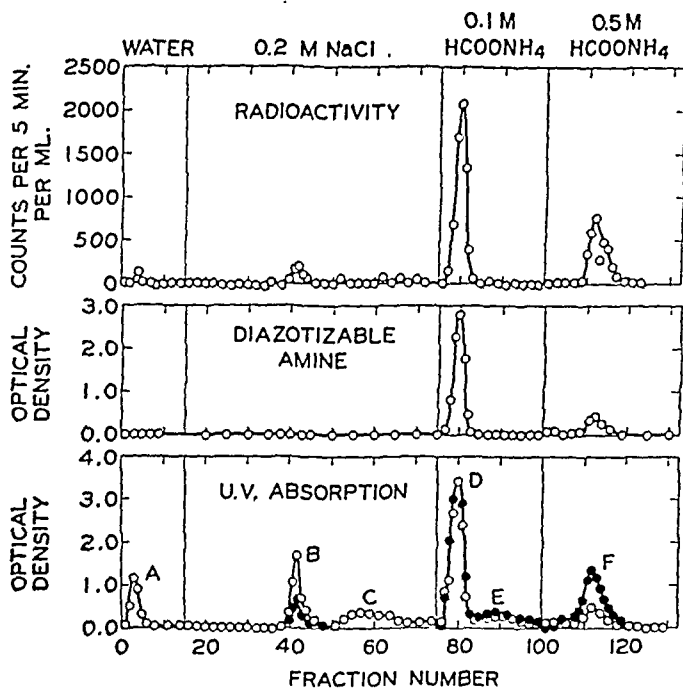

FIG. 3. Degradation of xanthine-8-C[14].   The following mixture was incubated in a stoppered tube at 35°: 80 μmoles of xanthine-8-C[14] containing 42,000 c.p.m., 400 μmoles of DEA buffer, pH 9.0, 100 μmoles of potassium phosphate, pH 9.0, 144 μmoles of cysteine, a cell extract of *C. cylindrosporum* equivalent to about 10 mg. of protein, and water to give a final volume of 8.0 ml.   After 20 minutes, 7.0 ml. of the incubation mixture, without deproteinization or pH adjustment, were placed on a *Dowex* 50 H^{+} column 12 cm. × 1 sq. cm.   The eluting mixtures used are indicated.   Fractions of 5 ml. were collected, and the optical density was determined at 260 mμ (O) and at 240 mμ (●).   Diazotizable amine and C[14] were determined on a 1 ml. aliquot of the eluate.   The letters refer to the components.

gether they generally accounted for 80 to 90 per cent of the diazotizable amine placed on the column, although the recovery in these particular experiments with the C[14]-labeled substrates is only 56 and 71 per cent. The relative amounts of each compound varied in different experiments, but the less basic Component D generally accounted for 80 per cent of the diazotizable amine recovered.

[4] Components A and E are unidentified.   Component B is unchanged xanthine. Component C was identified as 4-ureido-5-imidazolecarboxylic acid on the basis of absorption spectra and chromatographic behavior.

The large amounts of salts present in the fractions prevented a satisfactory comparison by paper chromatography of these compounds with the compound present in the original enzymatic incubation mixture. However, both Components D and F were decomposed by the cell extract when incubated at pH 7.0 (Fig. 5).

Since C-8 of xanthine was retained in the enzymatic intermediates, and since these compounds reacted in the Pauly test, it was concluded that both compounds contain the imidazole ring. Since C-2 of xanthine was not
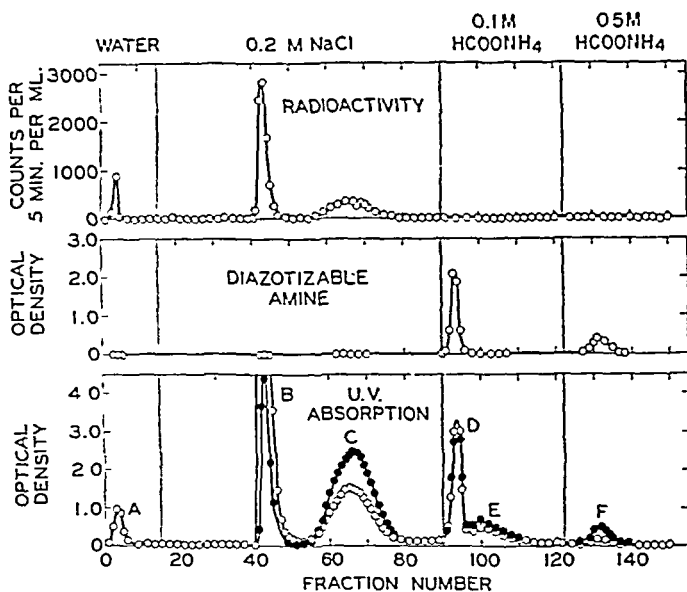

FIG. 4. Degradation of xanthine-2-C^{14}. The conditions are the same as for Fig. 3, except that 80 μmoles of xanthine-2-C^{14} containing 59,000 c.p.m. were used instead of xanthine-8-C^{14}.

retained in either compound, and since both reacted in the Bratton-Marshall test, it was concluded that both compounds contained a primary amino group substituted in position 4(5) of the imidazole nucleus. Unfortunately the fate of C-6 of xanthine was not determined, because of the unavailability of xanthine-6-C^{14}. The fact that 70 per cent of the diazotizable amine was retained on a Dowex 1 chloride column, suggested that the main component of the diazotizable amine fraction formed in the enzymatic reaction, Component D, contains a free carboxyl group. From the fact that C-6 of xanthine is finally converted to carbon dioxide in the fermentation (4) and the observation that 4-amino-5-imidazolecarboxamide was not metabolized, the structure of Component D was postulated as 4-amino-5-imidazolecarboxylic acid. Enzymatic or spontaneous decar-

boxylation of this compound would yield 4-aminoimidazole, which is possibly identical with Component F. Evidence in support of these conclusions follows.

Comparison of Enzymatic and Synthetic Compounds—4-Amino-5-imidazolecarboxylic acid and 4-aminoimidazole were synthesized as described.
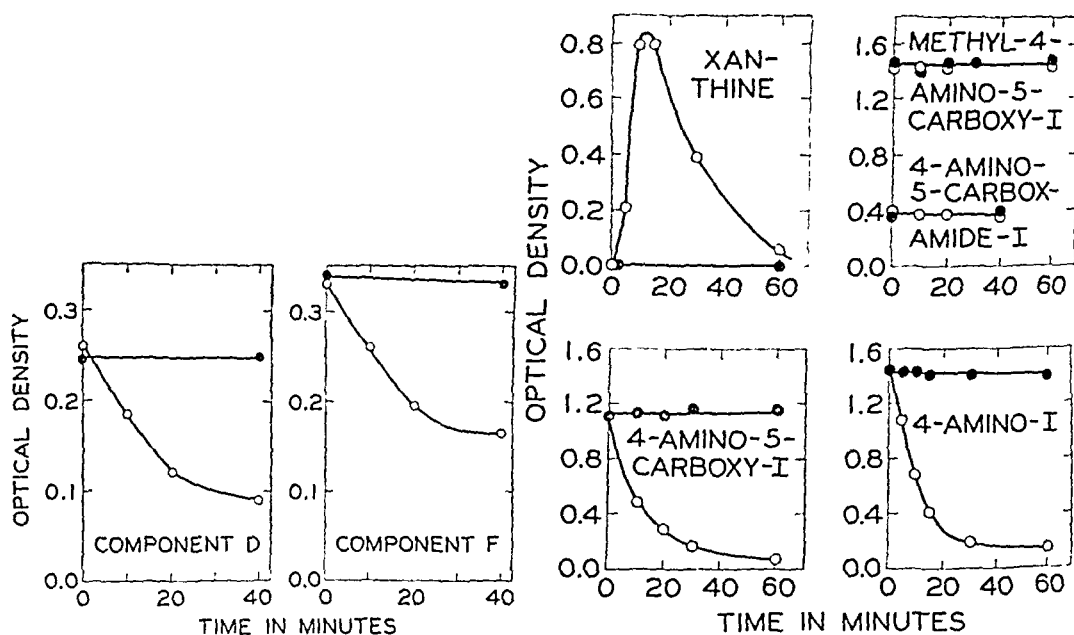

FIG. 5

FIG. 6

FIG. 5. Enzymatic decomposition of Components D and F. Stoppered tubes containing a solution of lyophilized Components D and F, 300 μmoles of TEA, pH 7.0, 0.5 ml. of the cell extract (O) or water (●), 72 μmoles of cysteine, and water to make the volume to 4.0 ml. were incubated at 35°. At the times indicated, 0.1 ml. was added to 5 ml. of 5 per cent perchloric acid, centrifuged, and analyzed by the Bratton-Marshall test.

FIG. 6. Enzymatic degradation of 4-amino-5-imidazolecarboxylic acid and 4-aminoimidazole. Stoppered tubes containing a solution of either synthetic aminoimidazole derivative or 40 μmoles of xanthine, 54 μmoles of cysteine, 200 μmoles of TEA, pH 7.0, and 0.5 ml. of a cell extract of *C. cylindrosporum* (O) or water (●) in a final volume of 4.0 ml. were incubated at 35°. At the times indicated, 0.1 ml. was removed, added to 5.0 ml. of 5 per cent perchloric acid, centrifuged, and analyzed by the Bratton-Marshall test.

The identification of these substances as intermediates in the enzymatic degradation of xanthine depends on (1) a demonstration of their enzymatic activity, (2) a comparison of the chromatographic behavior of the synthetic compounds with the products of the enzymatic reaction, and (3) a comparison of the spectral properties of the compounds and the derivatives formed in the Bratton-Marshall test. It has not been possible to isolate either compound as a crystalline substance from the synthetic or enzymatic reactions.

Both synthetic compounds when incubated with the cell extract at pH 7.0 were readily degraded to products which did not react in the Bratton Marshall test (Fig. 6). The observed rates of degradation were sufficiently great to account for the rate of disappearance of diazotizable amine observed with xanthine as a substrate. Neither the methyl ester nor the amide of 4-amino-5-imidazolecarboxylic acid was degraded.

TABLE II

Chromatography of Aminoimidazole Derivatives and Related Compounds

	Solvent 1*	Solvent 2*	Solvent 3*	Color in Pauly test
	R_F	R_F	R_F	
Enzyme incubation mixture†	0.42	0.13	0.19	Blue
4-Amino-5-imidazolecarboxylic acid.... ..	0.42	0.13	0.19	"
4-Aminoimidazole	0.43	0.35	‡	"
4-Amino-5-imidazolecarboxylic acid methyl ester§	0.72	0.61	0.84	Orange
4-Amino-5-imidazolecarboxamide‖	0.49	0.33	0 57	Blue
4-Hydroxy-5-imidazolecarboxamide¶	0.30	0.21		Red
4-Formamido-5-imidazolecarboxamide¶	0.52	0.36		Green
4-Guanidinoimidazole**	0.53	0.37		Purple
5-Imidazolecarboxylic acid††	0.30	0.15	0.49	Yellow
Xanthine .. .	0.40	0.25		Orange

* Solvent 1, n-propanol-1 N acetic acid (3:1) (11); Solvent 2, n-propanol-water (3:1); Solvent 3, acetone-2 M triethylamine-water (160:1:40).

† Conditions for the formation of the intermediate were the same as those described for Fig. 2 with DEA buffer at pH 9.0. Usually, two to four 0 01 ml. aliquots of the reaction mixture were spotted on paper without deproteinization or pH adjustment.

‡ The compounds were unstable in this solvent under the conditions used and gave no Pauly-positive material. Similar instability was noted on papers developed in n-propanol-1 N ammonium hydroxide (3:1).

§ A gift of Dr. H. K. Mitchell
‖ A gift of Dr. S. Gurin.
¶ A gift of Dr. E. Shaw.
** A gift of Dr. G. B. Brown.
†† A gift of Dr. R. G. Jones.

The identification of the amine formed from xanthine by the cell extract when incubated at pH 8.4 was complicated because of the instability of 4-amino-5-imidazolecarboxylic acid in acidic solvents and the instability of 4-aminoimidazole in basic solvents. Samples of the enzymatic incubation mixture, 4-aminoimidazole, and 4-amino-5-imidazolecarboxylic acid all showed a single component which gave a blue color in the Pauly test[5] and

[5] It may be noted that the blue diazo color is given only by the aminoimidazoles, although not all aminoimidazoles give a blue color.

had an R_F of 0.42 on paper chromatograms developed in *n*-propanol-acetic acid (Table II). However, when chromatographed in propanol-water or acetone-triethylamine, the enzymatic product behaved like 4-amino-5-imidazolecarboxylic acid and could be distinguished from 4-aminoimidazole. By eluting spots from chromatograms run in propanol-acetic acid and re-running them with propanol-water, it was shown that the enzymatic product and the synthetic 4-amino-5-imidazolecarboxylic acid had been converted to 4-aminoimidazole during chromatography in the acidic solvent. It is not clear whether Component F, seen on Dowex 50 H⁺ chromatography, is formed enzymatically or by the non-enzymatic decarboxylation of 4-amino-5-imidazolecarboxylic acid.
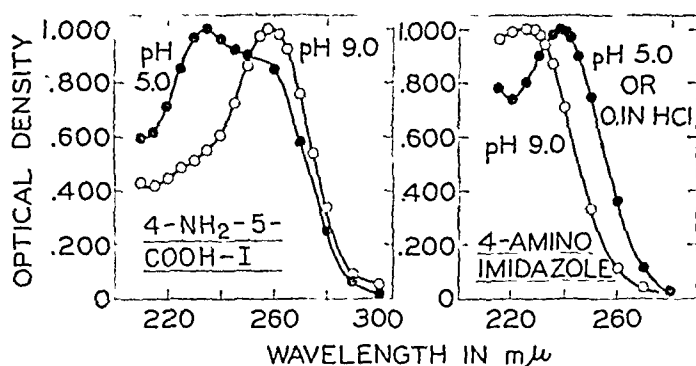

Fig. 7. Absorption spectrum of synthetic 4-amino-5-imidazolecarboxylic acid and 4-aminoimidazole. Substrates were freshly prepared as described, but the sodium sulfide was omitted from the solutions. Aliquots were diluted in 0.1 M sodium pyrophosphate (pH 9.0), 0.1 M KOH, or 0.025 M sodium acetate buffer (pH 5.0).

The absorption spectra of synthetic 4-amino-5-imidazolecarboxylic acid and 4-aminoimidazole are presented in Fig. 7. These curves are not strictly reproducible because of the instability of both compounds. Approximate values for the extinction coefficients were obtained by basing the compound concentrations on N determinations of the solutions. The molecular extinction coefficients of 4-amino-5-imidazolecarboxylic acid at the wavelength of maximal absorption were 9300 at 260 mμ at pH 9.0 and 7000 at 260 mμ in 0.1 M KOH. The value obtained for 4-aminoimidazole was 3800 at 238 mμ in 0.1 M HCl or at pH 5.0. The 260 to 240 mμ absorption ratios for 4-amino-5-imidazolecarboxylic acid and for 4-aminoimidazole at pH 5.0, 0.88 and 0.37, respectively, are in good agreement with the corresponding values of 0.8 and 0.4 found for Components D and F, respectively, in the chromatography of the enzymatic reaction mixture.

The spectra of the product formed in the Bratton-Marshall test with Component D and with the enzymatic product accumulated at pH 8.4 agree well with that of 4-amino-5-imidazolecarboxylic acid and show a

maximum at 502 mμ (Fig. 8). The maximal absorption of the product formed from 4-aminoimidazole occurs at 514 mμ. The spectra are easily
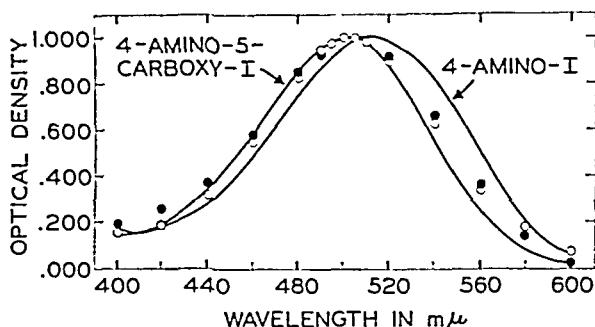

FIG. 8. Absorption spectra of the Bratton-Marshall reaction product of 4-amino-5-imidazolecarboxylic acid and 4-aminoimidazole. The Bratton-Marshall reaction was carried out on freshly prepared samples of the synthetic compounds as described. Spectra were determined with the reaction reagents in the reference cells. O, spectra of the compound formed from the amine accumulated at pH 8.4 under the conditions described in Fig. 2; ●, spectrum of the compound formed from Component D in this test. In recalculating the values, the maximal absorption is taken as 1.0

TABLE III

Enzymatic Conversion of 4-Amino-5-imidazolecarboxylic Acid to 4-Aminoimidazole

	Total μmoles		
Omission	Diazotizable amine	4-Amino 5-imidazolecarboxylic acid	4-Aminoimidazole
Enzyme, EDTA . .	4.0	3.0	0.5
" FeSO$_4$	3.9	2.9	0.5
EDTA	1.1	0.9	0.2
FeSO$_4$ . .	3.9	1.6	1.6

Stoppered tubes containing 200 μmoles of TEA, pH 8.0, 10 μmoles of cysteine, 0.2 ml. of the synthetic 4-amino-5-imidazolecarboxylic acid solution (equivalent to 4 μmoles), 0.4 ml. of the cell extract where indicated, 5 μmoles of EDTA, pH 8, or 0.5 μmole of ferrous sulfate, and water to make the volume 1.0 ml. were incubated at 37° for 5 minutes. 1 volume of ethanol containing 0.1 ml. of 2 M sodium sulfide per 10 ml. was added to the samples. The total diazotizable amine was determined on an aliquot of this sample, and 1 ml. was fractionated on Dowex 50 NH$_4$+ for the separation of the two imidazole derivatives as described.

distinguished from that given by 4-amino-5-imidazolecarboxamide, which has an absorption maximum at 518 mμ and a shoulder at the longer wavelengths under these conditions.

Enzymatic Decarboxylation of 4-Amino-5-imidazolecarboxylic Acid—The

conditions used for demonstrating the enzymatic conversion of 4-amino-5-imidazolecarboxylic acid to 4-aminoimidazole are based on the observation that certain metallic ions, such as Fe^{++} or Mn^{++}, are required for the enzymatic degradation of 4-aminoimidazole. It was therefore possible to inhibit the degradation of 4-aminoimidazole by the crude cell extract by the addition of ethylenediaminetetraacetic acid (EDTA) (Table III). The conditions of the assay were designed to minimize the non-enzymatic decarboxylation of the substrate; however, 12 per cent of the diazotizable amine originally added as 4-amino-5-imidazolecarboxylic acid was recovered as 4-aminoimidazole in the control tube and represents spontaneous decarboxylation of the substrate. On incubation of 4-amino-5-imidazolecarboxylic acid with the enzyme, but in the absence of EDTA, only 27 per cent of the diazotizable amine was recovered, demonstrating the enzymatic degradation of the substrate (2). In the presence of EDTA, 98 per cent of the diazotizable amine was recovered. Of this, 41 per cent could be accounted for as 4-amino-5-imidazolecarboxylic acid, while 41 per cent was recovered as 4-aminoimidazole.

DISCUSSION

4-Amino-5-imidazolecarboxylic acid was first mentioned in a proposed chemical synthesis of xanthine by Fargher and Pyman (8). Windaus and Langenbeck (10) later described the synthesis of 4-nitro-5-imidazolecarboxylic acid which on conversion to the ester or amide could be reduced to the corresponding aminoimidazole derivative with palladium and hydrogen, and Allsebrook et al. (9) using the methyl ester were then able to synthesize xanthine by the general method proposed by Fargher and Pyman. The free 4-amino-5-imidazolecarboxylic acid, however, has not been previously prepared and its formation in the anaerobic degradation of xanthine represents the first time the compound has been described.

Attempts to synthesize and isolate 4-aminoimidazole have been reported, but have generally been unsatisfactory (13–15). Reduction of 4-nitroimidazole with tin and hydrochloric acid resulted mainly in the formation of ammonia and glycine (13). The intermediates involved in the ring opening were not determined, but the process may bear a close relation to the enzymatic degradation of 4-aminoimidazole. The dihydrochloride of 4-aminoimidazole has been prepared and isolated by Hunter and Nelson (16) who used sodium amalgam for the reduction of 4-nitroimidazole and maintained anhydrous conditions to minimize hydrolysis of the base. The compound as described by these workers is extremely unstable, and evidence for the formation of dimers under conditions similar to those used in the reduction was reported (17).

It has been observed in this investigation that 4-nitroimidazole is reduced

catalytically in acidic or alkaline aqueous media with the theoretical amount of hydrogen uptake. However, the product formed in alkaline media is not degraded by cell extracts of *C. cylindrosporum* and gives a purple or blue color in the Bratton-Marshall test, instead of the pink color obtained with the product formed during acid reduction. It is possible that dimerization occurs under alkaline conditions to give products related to those described by Hunter and Hlynka (17).

Although the formation of 4-amino-5-imidazolecarboxylic acid and 4-aminoimidazole has been demonstrated in a degradative process, there is a strong possibility that the *N*-ribose phosphates of these compounds participate as precursors of 4-amino-5-imidazolecarboxamide ribotide in the biosynthesis of purines (18).

SUMMARY

Extracts of *Clostridium cylindrosporum* incubated with xanthine at pH 8.4 accumulate a diazotizable amine which has been identified as 4-amino-5-imidazolecarboxylic acid. The extracts also catalyze the decarboxylation of this compound to form 4-aminoimidazole. Both compounds have been prepared by the catalytic reduction of the respective nitroimidazole derivatives. The spectral and chromatographic properties of the compounds are described.

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PURINE FERMENTATION BY CLOSTRIDIUM CYLINDROSPORUM

IV. 4-UREIDO-5-IMIDAZOLECARBOXYLIC ACID*

By JESSE C. RABINOWITZ and W. E. PRICER, Jr.

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Xanthine (I) is converted to carbon dioxide, formic acid, glycine, and ammonia by crude extracts of *Clostridium acidi-urici* (1) and *Clostridium cylindrosporum* (2). Conditions under which an intermediate in this process is accumulated in incubation mixtures of xanthine and the crude extract were described in Paper III of this series (3). The compound was identified as 4-amino-5-imidazolecarboxylic acid (III), and its enzymatic decarboxylation to 4-aminoimidazole (IV) by extracts of *C. cylindrosporum* was also demonstrated.

The close relation of these compounds to 4-amino-5-imidazolecarboxamide is evident and suggested that this purine fermentation parallels the pathway of purine biosynthesis (4) involving the free bases rather than ribotides. However, 4-amino-5-imidazolecarboxamide is not degraded by extracts of the organism which convert xanthine to 4-amino-5-imidazolecarboxylic acid. Another intermediate in the degradative path which was found to accumulate when extracts were incubated with xanthine in the presence of sequestering agents is described in this paper. The isolation and characterization of this compound as 4-ureido-5-imidazolecarboxylic acid (II) provide additional evidence regarding the general scheme of purine degradation in *C. cylindrosporum*. This scheme, based on the observation presented here and in previous papers of the series (2, 3), is represented in the accompanying diagram.

EXPERIMENTAL

Methods—Strain HC1 of *C. cylindrosporum* was used. Stock cultures, cells, and extracts of the lyophilized cells were prepared as described previously (5). *Lactobacillus arabinosus*, ATCC No. 8014, was grown on the synthetic medium described by Craig and Snell (6), without added p-amino-benzoic acid or pyrimidines, supplemented with 100 γ of folic acid per 10 ml. of medium. A purineless mutant of *Escherichia coli*, 55-B-75, was kindly provided by Dr. B. D. Davis and grown under his conditions (7).

Whatman No. 1 paper was used for the paper chromatograms, which were developed by the ascending technique at room temperature for 15 hours with solvent mixtures described in the text. Compounds were detected on the chromatograms with the Pauly reagents (8), the Bratton-Marshall reagents (9), or by inspection under a Mineralight lamp (10).

Absorption spectra were determined in 1 cm. cells with the Beckman model DU spectrophotometer equipped with a photomultiplier.

4-Amino-5-imidazolecarboxylic acid was determined by the test for diazotizable amines described by Bratton and Marshall (11), but 5 per cent perchloric acid was substituted for the trichloroacetic acid used in the original method (3).

XANTHINE DEGRADATION BY EXTRACTS OF CLOSTRIDIUM CYLINDROSPORUM
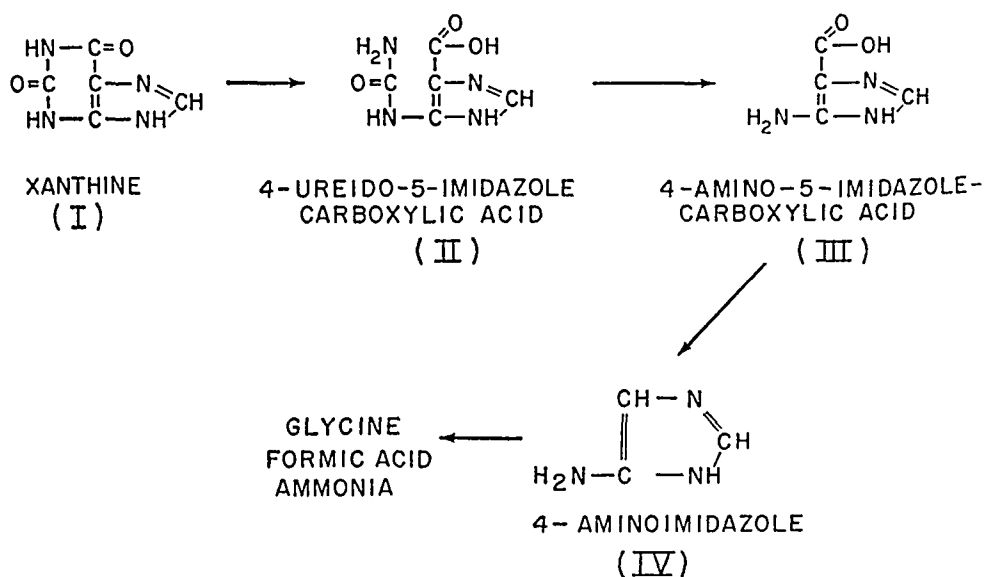
4-Ureido-5-imidazolecarboxylic acid, when present as the only imidazole derivative, was determined by a modification of the Pauly test (12). The color formed is unstable, but the maximal optical density at 520 mμ is proportional to the amount of 4-ureido-5-imidazolecarboxylic acid. The maximal color is attained 0.5 to 2.0 minutes after mixing sample and reagents.

4-Aminoimidazole fails to react in the Pauly test after treatment with nitrous acid, while the reactivity of 4-ureido-5-imidazolecarboxylic acid is unaffected. The following procedure, therefore, was used to determine 4-ureido-5-imidazolecarboxylic acid when it occurred together with 4-aminoimidazole. The deproteinized sample is diluted to 0.7 ml. with water and treated with 0.1 ml. of 5 per cent perchloric acid and 0.1 ml. of 0.1 per cent sodium nitrite. After 2 minutes, 0.1 ml. of 0.5 per cent ammonium sulfamate is added. The imidazole is determined by the modified Pauly test just described.

Protein was determined by a turbidimetric method (13).

Materials—4-Aminoimidazole was prepared from 4-nitroimidazole by catalytic reduction (3).

4-Ureidoimidazole was prepared as described by Hunter and Hlynka (14). A solution containing 7 mmoles of 4-aminoimidazole, 14 mmoles of potassium cyanate, and 14 mmoles of hydrochloric acid in 420 ml. of water was kept at room temperature overnight. The mixture was adsorbed on Dowex 50 H⁺ and eluted with 5 N HCl. The HCl was removed under reduced pressure. The crystalline hydrochloride of 4-ureidoimidazole was obtained on addition of ethanol to a water solution of the compound. The product was recrystallized from ethanol-water. M.p. 209–210° (210° (14)). $C_4H_7N_4OCl$, calculated, C 29.6, H 4.3 per cent; found, C 29.44, H 4.48 per cent.

Diethanolamine (DEA) and triethanolamine buffers were prepared by neutralization of the free bases with hydrochloric acid. Xanthine, obtained from the Nutritional Biochemicals Corporation, and commercial samples of inorganic salts were used without further purification.

Results

Formation—Xanthine, incubated with cell-free extracts of *C. cylindrosporum* at pH 9.0, is converted to 4-amino-5-imidazolecarboxylic acid (3). When the incubation is carried out in the presence of α,α'-dipyridyl or potassium cyanide, a decreased amount of 4-amino-5-imidazolecarboxylic acid is formed, and another product can be detected which reacts to form a magenta color when sprayed with the Pauly reagents. 4-Amino-5-imidazolecarboxylic acid gives a blue color in this test. Both compounds may also be detected as quenching areas when viewed with the Mineralight lamp. In contrast to 4-amino-5-imidazolecarboxylic acid, the second product formed from xanthine does not react with the Bratton-Marshall reagents, indicating the absence of a diazotizable amino group.

The products formed from xanthine in the presence of various sequestering agents were determined (Fig. 1). In the absence of sequestering agent, 4-amino-5-imidazolecarboxylic acid is the only product which can be detected. In the presence of 1×10^{-3} M ethylenediaminetetraacetic acid (EDTA), the formation of 4-amino-5-imidazolecarboxylic acid is completely inhibited, while the maximal amount of another imidazole, stable to nitrous acid, is formed. Dipyridyl and potassium cyanide are required in higher concentrations to produce these effects.

Isolation—For isolation of the intermediate, the following mixture was incubated at 37° under anaerobic conditions: 13 mmoles (2 gm.) of xanthine, 6 mmoles of EDTA adjusted to pH 9.0, 12 mmoles of cysteine adjusted to pH 9.0, 120 mmoles of DEA at pH 9.0, an extract of *C. cylindrosporum*

equivalent to 200 mg. of protein, and water to make the volume 900 ml. After 4 hours, the amount of intermediate formed, as determined in the colorimetric test, had reached a maximum. Perchloric acid was then added to give a final concentration of 1 per cent. The precipitated protein was removed by centrifugation.
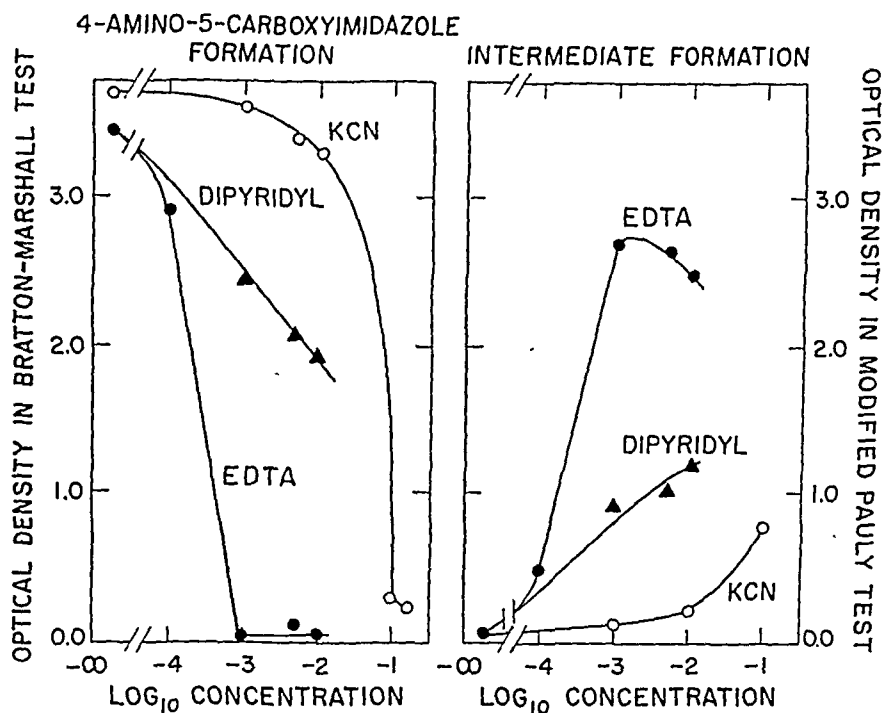

FIG. 1. Effect of sequestering agents on xanthine decomposition by *C. cylindrosporum* extracts. The incubation mixtures contained 40 μmoles of xanthine, 40 μmoles of cysteine, 400 μmoles of DEA, pH 9.0, 0.4 ml. of bacterial extract, sequestering agent as indicated, and water to make the volume 4.0 ml. The tubes were stoppered and incubated for 1 hour at 37°. Left-hand section, optical density at 510 mμ of 0.1 ml. aliquot in the Bratton-Marshall test. Right-hand section, optical density at 520 mμ of 0.1 ml. aliquot in the modified Pauly test after treatment with sodium nitrite as described under "Methods."

A precipitate formed in the acidified supernatant solution on storage at 2° overnight. This precipitate was separated by centrifugation, dissolved in 0.1 M NH$_4$OH, and analyzed spectrophotometrically. It was found to contain 3.2 mmoles of xanthine and 2.3 mmoles of the intermediate and was discarded. The supernatant solution which contained 0.7 mmole of xanthine and 6.5 mmoles of the intermediate was chromatographed on Dowex 50 H$^+$ (Fig. 2). The intermediate was detected in fractions eluted with 30 to 43 column volumes. These fractions were combined, and the volume was reduced from 1400 to 750 ml. under reduced pressure. The solution, containing 5.8 mmoles of intermediate and 0.15 mmole of xanthine, was
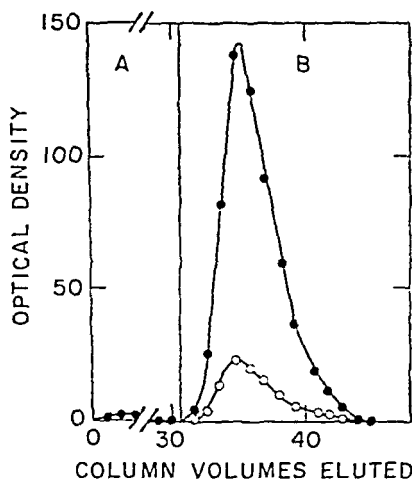
FIG. 2. Chromatography of reaction mixture on Dowex 50 H+. The deproteinized reaction mixture (930 ml.) was passed over a resin column 12 cm. × 8.3 sq. cm., and the column developed by gradient elution. In A, a reservoir containing 0.10 M ammonium hydroxide and a 2 liter mixing chamber of water were used. In B, the stock eluent solution was replaced with 0.30 M ammonium hydroxide. The optical density of the fractions was determined in 0.1 M sodium pyrophosphate, pH 9.0, at 250 mμ (●) and at 280 mμ (○).

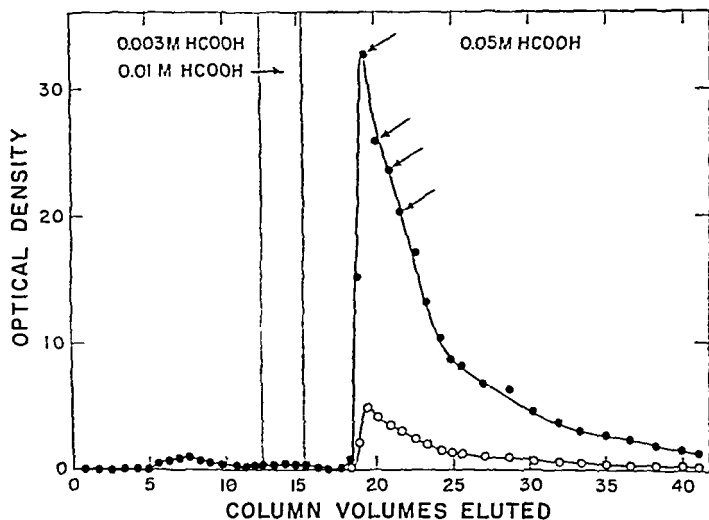FIG. 3. Chromatography of the Dowex 50 eluate on Dowex 1 formate. The Dowex 50 eluate (750 ml.) was placed on a 10 cm. × 30 sq. cm. column of Dowex 1 formate. Formic acid of the molarity indicated was used for elution. The optical density of the fractions was determined in 0.1 M sodium pyrophosphate, pH 9.0, at 260 mμ (●) and at 240 mμ (○). Fractions which contained crystalline material are indicated by the arrows.

then chromatographed on Dowex 1 formate (Fig. 3). Several fractions emerged from the column, containing crystalline material. These crystals

TABLE I

Chromatography of Enzymatic Product and Some Related Imidazoles on Paper

Sample	Solvent		
	Pro-NH₃*	MCF†	Pro-HOAc‡
	R_F	R_F	R_F
Enzymatic product....................................	0.30	0.50	0.29
" " heated with quinoline...........	0.50	0.61	0.44
4-Amino-5-imidazolecarboxylic acid.................	0.21	0.62	0.42
" " + HCNO.....	0.31	0.51	0.29
	0.50	0.60	0.46
4-Ureidoimidazole..................................	0.50	0.61	0.44

* n-Propanol 3 parts, 1 M ammonium hydroxide 1 part (8).
† Methanol 3 parts, chloroform 3 parts, 10 per cent formic acid 1 part.
‡ n-Propanol 3 parts, 1 M acetic acid 1 part (8).

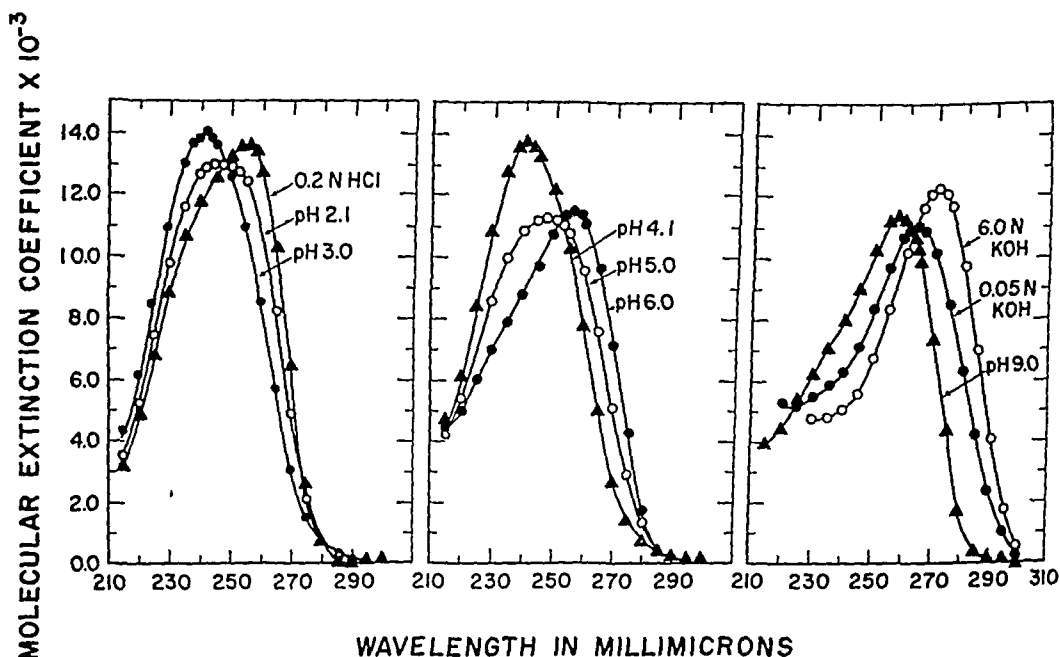FIG. 4. Spectra of isolated enzymatic product. Spectra at pH 2, 3, 4, and 5 were determined in 0.1 M potassium phosphate adjusted to the pH indicated. Curves at pH 6 and 9 were determined in 0.1 M sodium pyrophosphate.

were removed by centrifugation. The supernatant solutions and fractions eluted with 18 to 42 column volumes were concentrated under reduced pressure to about 20 ml. The crystalline material which formed was sepa-

rated by centrifugation, washed with cold water, and recrystallized from water. A total of 730 mg. of material was isolated, which was calculated to represent 3.5 mmoles of the anhydrous intermediate on the basis of the absorption at 260 mμ.

Identification—The isolated material was converted to 4-amino-5-imidazolecarboxylic acid by the extract; it gave a positive Pauly test, indicating the presence of an imidazole ring, a negative Bratton-Marshall test, indicating the absence of a primary amino group, and a positive carbamyl test (15), and was adsorbed on both cationic and anionic ion exchangers. These observations suggested 4-ureido-5-imidazolecarboxylic acid as a possible structure of the isolated material.

The isolated material contained a variable amount of water of crystallization. The elementary analysis of this material, dried to constant weight at 100°, agrees fairly well with that of 4-ureido-5-imidazolecarboxylic acid: $C_5H_6O_3N_4$, calculated, C 35.30, H 3.55, N 32.93 per cent; found, C 35.02, H 3.89, N 32.04 per cent.

Koritz and Cohen (15) have described a colorimetric test for carbamyl compounds in which carbamylglutamic acid gives 0.086 times the color of an equimolar amount of citrulline. The isolated material and synthetic 4-ureidoimidazole gave, respectively, 0.062 and 0.089 times the color of an equimolar amount of citrulline in this test.

Further evidence concerning the chemical nature of the intermediate was obtained by treating the isolated compound with cupric acetate in quinoline at 200°, conditions commonly employed for the decarboxylation of aromatic carboxylic acids (16). The product formed could not be distinguished from a synthetic sample of 4-ureidoimidazole on paper chromatograms in three solvent systems (Table I).

Synthesis of a compound having the same chromatographic properties as the isolated compound was accomplished by treating 4-amino-5-imidazolecarboxylic acid with potassium cyanate under the same conditions employed in the synthesis of 4-ureidoimidazole from 4-aminoimidazole (14). A second product was formed during this reaction which could not be distinguished from 4-ureidoimidazole in any of the solvent systems tested. The formation of this product is not unexpected under the acid conditions used, since 4-amino-5-imidazolecarboxylic acid decarboxylates readily below pH 7 (3).

Physical Properties—The absorption spectrum and extinction coefficients of the isolated enzymatic product, 4-ureido-5-imidazolecarboxylic acid, are given in Fig. 4. The molecular extinction coefficients at the wave-length of maximal absorption were found to be 13,800 at 255 mμ in 0.2 N HCl, 14,300 at 242 mμ from pH 3 to 4, 11,700 at 256 mμ from pH 6 to 9, 12,600 at 270 mμ in 6 N KOH.

The pK_a' values calculated from the absorption spectra, which were not obtained at constant ionic strengths, are 2.0, 4.9, and 12.2.

The solubility of the isolated material in water is 1.5 μmoles per ml. at 35.5°, 1.0 μmole per ml. at 23°, and 0.6 μmole per ml. at 0°.
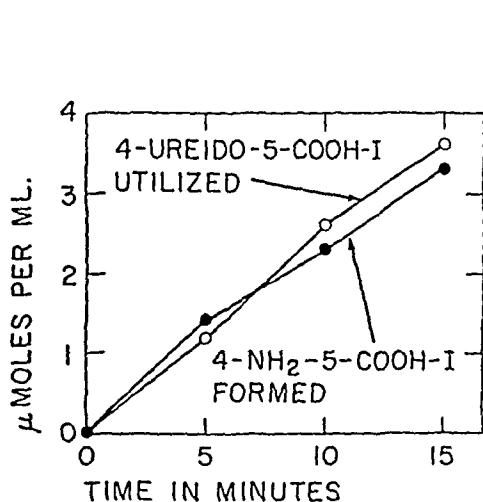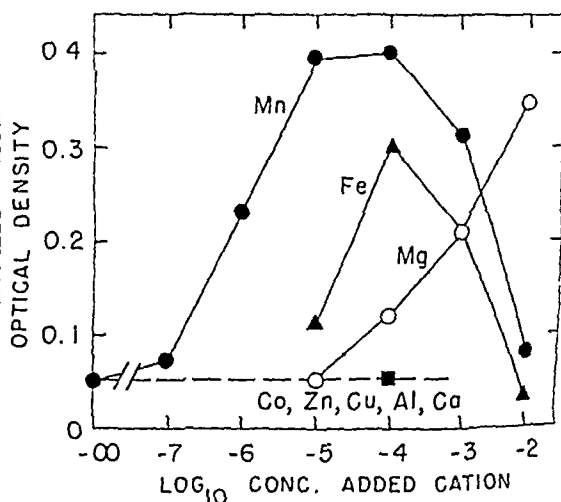

FIG. 5

FIG. 6

FIG. 5. Conversion of intermediate to 4-amino-5-imidazolecarboxylic acid by extracts of *C. cylindrosporum.* 20 μmoles of intermediate (4-ureido-5-imidazole-carboxylic acid), 40 μmoles of cysteine, 400 μmoles of DEA, pH 9.0, 0.1 ml. of extract (1.3 mg. of protein), 2 μmoles of ferrous sulfate, and water to make the volume 4.0 ml. were incubated in stoppered tubes at 37°. Samples (0.5 ml.) were added to an equal volume of 5 per cent perchloric acid. Aliquots were assayed as described under "Methods."

FIG. 6. Effect of cations on the conversion of 4-ureido-5-imidazolecarboxylic acid to 4-amino-5-imidazolecarboxylic acid. Stoppered tubes containing 2.5 μmoles of 4-ureido-5-imidazolecarboxylic acid, 2.0 μmoles of cysteine, 200 μmoles of DEA, pH 9.0, an extract of *C. cylindrosporum* equivalent to 0.65 mg. of protein, additions of $MnCl_2$, $FeSO_4$, $MgCl_2$, $CoCl_2$, $ZnSO_4$, $Cu(OOCCH_3)_2$, $Al_2(SO_4)_3$, $CaCl_2$ as indicated, and water to make the total volume 2.0 ml. were incubated for 15 minutes at 37°. The deproteinized samples were analyzed for 4-amino-5-imidazolecarboxylic acid by the Bratton-Marshall test.

Biological Activity—Reincubation of the isolated 4-ureido-5-imidazolecarboxylic acid with the extract in the absence of sequestering agent results in formation of 4-amino-5-imidazolecarboxylic acid (Fig. 5).

The conversion of 4-ureido-5-imidazolecarboxylic acid to 4-amino-5-imidazolecarboxylic acid by the extract is stimulated by the addition of Mn^{++}, Fe^{++}, and Mg^{++} (Fig. 6). The ions active in stimulating the crude system also reactivated the system completely inhibited by EDTA.

The ability of 4-ureido-5-imidazolecarboxylic acid to replace xanthine was studied with *L. arabinosus.* Growth of this organism depends on a source of added purines in a medium containing folic acid but free of py-

rimidines and *p*-aminobenzoic acid (17).   Xanthine can satisfy the purine requirement of the organism under these conditions (Fig. 7).   However, 4-ureido-5-imidazolecarboxylic acid was ineffective under the same conditions.   It also failed to inhibit growth of this organism in the presence of suboptimal amounts of xanthine.

A purineless *E. coli* mutant, 55-B-75, which can utilize adenine, guanine, hypoxanthine, their ribosides or ribotides, and xanthine[1] to satisfy its purine requirement also failed to grow on a medium supplemented with 4-ureido-5-imidazolecarboxylic acid.[2]
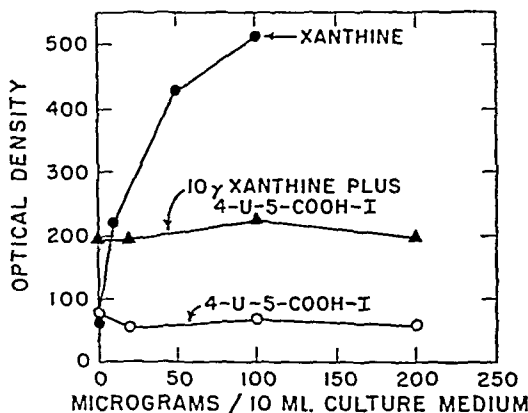
FIG. 7. Growth of *L. arabinosus* on xanthine and 4-ureido-5-imidazolecarboxylic acid.   The cultures were incubated for 24 hours at 37°.   The optical density was determined at 660 mμ with the Coleman junior spectrophotometer.

DISCUSSION

Extracts of *C. cylindrosporum* convert xanthine to an imidazole derivative which can be caused to accumulate in the presence of a sequestering agent.   This product has been identified as 4-ureido-5-imidazolecarboxylic acid.   The synthesis of 4-ureido-5-imidazolecarboxylic acid has been reported by Allsebrook, Gulland, and Story (18) by alkaline hydrolysis of the reaction product of potassium cyanate and methyl-4-amino-5-imidazolecarboxylic acid.   A small sample of this product was kindly sent to us by Professor D. O. Jordan.   The sample had undergone obvious deterioration, since on examination by us, 11 years after it was synthesized, it was found to contain 29 per cent xanthine in addition to an imidazole component.   The spectrum and behavior on ion exchange columns of the imidaz-

[1] Dr. B. D. Davis, personal communication.

[2] This compound was also tested by Professor J. Gots of the University of Pennsylvania and was unable to satisfy the purine requirement of fifteen other *E. coli* mutants.

ole component were similar to those of the enzymatic product, although the two substances could be separated on paper chromatograms in propanol-1 M acetic acid (3:1). Sufficient material was not available to us for further examination.

The biochemical transformations which xanthine has been shown to undergo are limited to its oxidation to uric acid (19), its reduction to hypoxanthine (20), its conversion to the deoxynucleoside and the nucleoside (21), and the reaction described in the present paper, its apparent hydrolysis to 4-ureido-5-imidazolecarboxylic acid. The latter reaction has not been described previously. However, this cleavage is similar to such known reactions as the conversion of allantoin to allantoic acid (22), the presumed hydrolysis of barbituric acid to ureidomalonic acid (23), and the hydrolysis of dihydroorotic acid to ureidosuccinic acid (24), which also involve the conversion of the structure NH—CO—NH—CO to NH—CO—NH$_2$ COOH.

The isolation of 4-ureido-5-imidazolecarboxylic acid as a product of xanthine degradation was facilitated by the fact that its further degradation by *C. cylindrosporum* extracts is inhibited by sequestering agents. Mn^{++} and Fe^{++} were most effective in activating the unpurified extract or in reversing the inhibition caused by EDTA. However, the determination of the metal requirements must await purification of the enzyme. Ureidosuccinase, which also catalyzes the hydrolysis of a ureido group, has been shown to require Mn^{++} or Fe^{++} for activity (24).

Treatment of 4-ureido-5-imidazolecarboxylic acid with hydrochloric acid at elevated temperature has been reported to cause ring closure, with the formation of xanthine (18). Attempts to demonstrate an analogous biological reaction, by replacing the xanthine requirement of *L. arabinosus* or a variety of *E. coli* mutants with 4-ureido-5-imidazolecarboxylic acid, have not been successful. It is possible that the nucleoside or nucleotide of the compound is required for the biological reaction. Thus, although most organisms cannot utilize 4-amino-5-imidazolecarboxamide for growth (25), it has been shown that the ribotide of this compound is converted to inosinic acid (26).

Despite the over-all similarities in the anabolic pathway leading to purine biosynthesis and the catabolic pathway (5), they are differentiated by the demonstration of the rôle of 4-ureido-5-imidazolecarboxylic acid as an intermediate in the latter process. This compound represents a cleavage between atoms 1 and 6 of the purine ring, while the intermediate in biosynthesis represents a cleavage between atoms 1 and 2.

SUMMARY

Xanthine, incubated with extracts of *Clostridium cylindrosporum* in the presence of sequestering agents, is converted to 4-ureido-5-imidazolecarbox-

ylic acid.   4-Ureido-5-imidazolecarboxylic acid is converted to 4-amino-5-imidazolecarboxylic acid by the extracts on the addition of the ions, Mn^{++} or Fe^{++}.   The position of this compound as an intermediate in xanthine degradation by *C. cylindrosporum* is thus established.

Conditions for the formation of 4-ureido-5-imidazolecarboxylic acid and the method used in its isolation are described.   The spectra and solubility of the compound were determined.

We wish to thank Dr. W. C. Alford for the microanalyses.

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THE OXIDATION OF TARTARIC ACID BY AN ENZYME SYSTEM OF MITOCHONDRIA*

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The rôle of certain hydroxy acids such as malic and lactic acids in animal, plant, and microbial metabolism is generally recognized. It was shown recently that mammalian liver contains a specific enzyme which oxidizes another hydroxy acid, glycolic acid (1). A similar glycolic acid oxidase was found in plant tissues (2). Relatively little is known, however, about the metabolism of a number of other hydroxy acids, such as tartaric, dihydroxyfumaric, hydroxypyruvic, and tartronic acids. Banga and Szent-Györgyi (3) and Banga and Philippot (4) extracted dihydroxyfumaric acid oxidase from plants. The oxidation product was believed to be diketosuccinic acid, although its identity has not been established. Further information on certain enzymatic reactions of dihydroxyfumaric and diketosuccinic acids in plant tissues was recently obtained by Stafford, Magaldi, and Vennesland (5). The rôle of these acids in animal metabolism was hitherto only suggested but not proved by experimental work. As early as 1912 Parnas and Baer (6) proposed a series of reactions, presumably occurring in muscle, which involved CO_2 fixation into hydroxypyruvic acid to yield the keto form of dihydroxyfumaric acid. The experimental evidence for such a reaction was, however, missing. Stepanow and Kusin (7) observed glycogen synthesis in muscle from dihydroxyfumaric acid. This work was extended to hydroxypyruvic acid by Akabori, Uehara, and Muramatsu (8) who were primarily interested in sugar synthesis in muscle tissue.

The purpose of this paper is to describe an enzyme system which acts upon tartrate. The emphasis is on the qualitative and quantitative characterization of the sequence of enzymatic reactions, while the components of the enzyme system are the subject of further studies.

Results

Occurrence and Properties of Enzyme System—The tartrate oxidizing enzyme system was found in the mitochondrial fraction of rat and beef tissue homogenates of all organs so far investigated.   Conditions for enzymatic oxidation of tartrate are established when diphosphopyridine nucleotide (DPN) and Mg^{++} (or Mn^{++}) are added to a suspension of sucrose mitochondria with D(—)- or *meso*-tartrate as substrate.   In intact mitochondria the cytochrome system is utilized as the terminal electron transfer mechanism to molecular oxygen.   Highest rates of O_2 uptake are obtained when the reaction is carried out at pH 8 to 8.3.   The enzyme system was brought

TABLE I

Comparison of "Apparent Tartaric Acid Oxidase" Activity of Various Tissues

Tissue	Activity, μl. O_2 per mg. protein per hr.	
	D(—)-	*meso*-
Rat liver Mw	10.0	4.6
" kidney Mw	16.4	9.6
" brain Mw .. .	6.2	3.1
" heart "	20.0	11.4
Beef heart Mw extract	74.0	106.0
Rat liver Mw extract	19.2	30.8

The manometric test system contained in a volume of 3 ml. 30 μmoles of tartrate, 50 μmoles of Tris, pH 8.3, 15 μmoles of Mg^{++}, and 1 mg. of DPN.   With intact mitochondria (Mw) 2 mg. of cytochrome c and in extracts 2 mg. of phenazine methonium sulfate were used as the carrier.   Temperature, 37°; gas phase, air; center well, 0.1 ml. of 20 per cent KOH + filter paper.   Time of reaction, 60 minutes.

into solution by either of two methods.   Mitochondria prepared by the isotonic sucrose procedure of Schneider (9) were treated with cold (—10°) acetone as described by Drysdale and Lardy (10).   The resulting dry powder was stirred for 1 hour at 0° with 0.1 M tris(hydroxymethyl)amino-methane (Tris) buffer of pH 8.3 and centrifuged (6000 $\times$ g for 30 minutes). As a routine the extract of 1 gm. of acetone powder with 10 ml. of buffer, containing 5 to 10 mg. of protein per ml., was used in most experiments. The enzyme system was also made soluble by extraction of freshly prepared mitochondria according to a method described by Kun (11).   The resulting extract was frozen and dried *in vacuo* after high speed centrifugation. When mitochondrial extracts were tested for "tartaric acid oxidase" activity, besides DPN and Mg^{++} a carrier dye, phenazine methonium sulfate, was required as a link to molecular oxygen.

Table I summarizes comparative data of "tartaric acid oxidase" activi-

ties of various tissues as well as of mitochondrial extracts as determined by the manometric procedure. The term "apparent tartaric acid oxidase" is merely used for convenience and indicates the sum total of several consecutive reactions measured under conditions in which maximal O_2 uptake is obtained. When intact mitochondria are in contact with the $D(-)$ or meso form of tartaric acid, the $D(-)$ form is oxidized at a greater rate. Aging of mitochondria, treatment with H_2O, or extraction of the enzyme system from the particulate form into solution reverses the apparent affinity, and in each case *meso*-tartrate is oxidized preferentially. This difference between the structurally bound and the soluble system may be explained by selective permeability of mitochondria. $L(+)$-Tartrate is not oxidized by any of the preparations. The tartrate oxidizing enzyme system is fairly stable; its activity, which diminishes to 50 to 60 per cent in 1 to 2 days (in an ice bath), can be almost completely restored by the addition of Mg^{++}.

Products of Tartrate Oxidation—Analyses of the deproteinized reaction mixtures resulted in the characterization of a number of keto acids, which were isolated as the 2,4-dinitrophenylhydrazine derivatives by means of a chromatographic procedure, described under "Methods" and reported in detail in another publication (12). The same products were formed by either intact mitochondria or extracts.

The identity of the keto acids was based upon the following criteria: (*a*) Rechromatography of a mixture of the synthetic pure compound together with the derivative isolated from enzyme incubation did not result in separation into two components. (*b*) The catalytic reduction of the 2,4-dinitrophenylhydrazine derivatives of both samples yielded the same amino acids, which were identified by the chromatographic method as described by Levy and Chung (13).

The chromatographic separation of the 2,4-dinitrophenylhydrazine derivatives of the keto acids formed during the enzymatic oxidation of tartrate is shown in Fig. 1. Compound 1 is the 2,4-dinitrophenylhydrazone of oxaloglycolic acid (the keto form of dihydroxyfumaric acid). Both the synthetic and enzymatic products yield upon hydrogenolysis hydroxyaspartic acid as the major amino acid and small amounts of serine, the latter as the result of decarboxylation. Both enzymatically formed and synthetic products were decarboxylated by refluxing in water at 100° for 1 hour to the bis-2,4-dinitrophenylhydrazine derivative of hydroxypyruvic acid. Compound 2 is the bis-2,4-dinitrophenylhydrazone (osazone) of diketosuccinic acid, which was also identified as diaminosuccinic acid. Compound 3 is the 2,4-dinitrophenylosazone of hydroxypyruvic acid, which upon reduction yielded a small amount of alanine and 2,3-diaminopropionic acid as the major product. Compound 4 separated into two com-

ponents which were identified as the isomers of the 2,4-dinitrophenylhydrazone of glyoxylic acid. The two components, also obtained with pure synthetic 2,4-dinitrophenylhydrazone of glyoxylic acid, were eluted separately and converted to the corresponding amino acid, which was glycine. The cis and trans isomers of oxaloglycolic acid 2,4-dinitrophenylhydrazone did not occur, provided all chemical operations were kept below 50°.

The *quantitative distribution* and the rate of keto acid formation from tartrate are reported in Table II and Fig. 2. Oxygen consumption, CO_2 evolution, and glyoxylic acid production from both D($-$)- and *meso*-tartrate are increased by Mg^{++}, while a simultaneous diminution of oxalo-
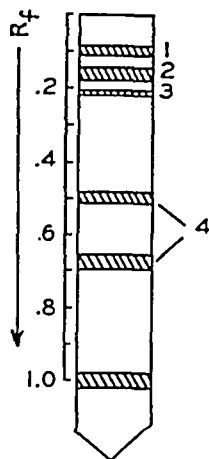
FIG. 1. The distribution of keto acid derivatives on a descending chromatogram. Relative values (R_F) were determined by taking the alkaline decomposition product of 2,4-dinitrophenylhydrazine as $R_F = 1$ (lowest line). Compound 1 is oxaloglycolic, Compound 2 diketosuccinic, Compound 3 hydroxypyruvic, Compounds 4 and 5 glyoxylic acid derivatives (see the text).

glycolate takes place. Hydroxypyruvate from *meso*-tartrate is doubled in the presence of Mg^{++} (Experiment A). Further studies dealing with the stability of keto acids produced during the enzymatic oxidation of tartrate revealed that diketosuccinate is non-enzymatically decarboxylated to tartronate, which was identified as its oxidation product, *i.e.* mesoxalic acid. This was isolated as the 2,4-dinitrophenylhydrazone. As shown in Table III, ethylenediaminetetraacetate (Versene) retards this very rapid decarboxylation. When Versene is present during the enzymatic oxidation of tartrate (Table II, Experiment B), oxaloglycolate and glyoxylate accumulate, while CO_2 evolution diminishes, as determined in other experiments. The metal ion-catalyzed decomposition of diketosuccinate thus markedly decreased the yield of total keto acids from tartrate. It was found in experiments with intact mitochondria (oxidation via the cytochrome system) that the main sequence of enzymatic reactions is the oxidation of tartrate

to oxaloglycolate, which then yields glyoxylate, while the oxidation to diketosuccinate and its decarboxylation to tartronate are considered as probably non-enzymatic side reactions.   An additional interfering reaction

TABLE II

Distribution of α-Keto Acids Produced from Tartrate (in μmoles)

Keto acid	Experiment A				Experiment B	
	No Mg^{++}		+ Mg^{++}		No Versene	+ Versene (0.01 M)
	D(—)-	meso-	D(—)-	meso-		
Oxaloglycolic...........	0.196	0.378	0.072	0.154	0.550	0.684
Diketosuccinic.........	0.018	0.026	0.008	0.014	0.026	0.028
Hydroxypyruvic.......	0.006	0.010	0.008	0.022	0.008	0.018
Glyoxylic..............	0.037	0.072	0.090	0.182	0.109	0.278
Total keto acids......	0.257	0.486	0.178	0.372	0.693	1.008
O₂ absorbed.........	0.535	1.680	1.780	2.760	1.300	1.300
CO₂ produced........	0.450	0.870	1.45	3.000		

Time of reaction, 15 minutes; temperature, 30°.   In Experiment B no Mg^{++} was added; the substrate was *meso*-tartrate.   Conditions as described in Table I.   Each flask contained 0.5 ml. of extract of beef heart mitochondria (4 to 7 mg. of protein).
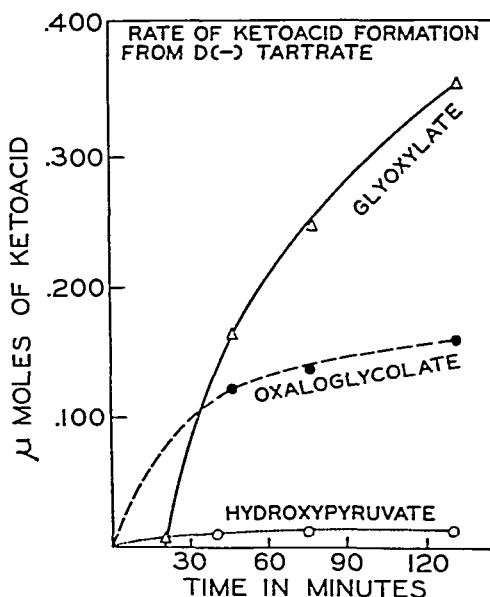

FIG. 2. The enzyme system was the same as that described in Table II.   Substrate, D(−)-tartrate; 15 μmoles of MgSO₄ were added.   Temperature, 30°.

which obscures the balance of products is the secondary oxidation of glyoxylate by H_2O_2, as discussed elsewhere (1, 2). Analyses for simultaneous disappearance of tartrate showed that 1.4 to 1.6 μmoles of tartrate were used up while 1 μmole of O_2 was absorbed. Unfortunately the analytical method for D(−)-tartrate by means of its complex formed with metavanadate (14) is not sufficiently accurate and sensitive to permit us to rely on it beyond an error of ±20 per cent.

It is interesting, but as yet unexplained, that the stoichiometric mixture of Versene + Mg^{++} (final concentration 10^{-2} M) caused complete inhibition of tartrate oxidation.

TABLE III

Decomposition of Diketosuccinate at pH 8.3

Time	Amount recovered	
	In Tris	In Tris + Versene (10^{-2} M)
min.	*per cent*	*per cent*
5	20 −35	43–50
10	1 − 5	36–40
15	0.1– 0.5	27–30
20	0	15
30	0	12

1 μmole of diketosuccinate was added at zero time, and the remaining keto acid was analyzed at the indicated times. The volume of reactants and the amount of Tris buffer were the same as in the enzymatic experiments. Temperature, 30°. No Mg^{++} was added.

Reversibility of Dehydrogenation of Tartrate—Studies concerned with the reversibility of the reactions catalyzed by the tartrate oxidizing enzyme system were confined to the first step of dehydrogenation to oxaloglycolate. The reduction of oxaloglycolate was measured as follows: crystalline yeast alcohol dehydrogenase (130,000 units) (15) was incubated at 30° for 10 minutes with 2 mg. of DPN and 0.05 ml. of 95 per cent ethanol in the presence of 20 μmoles of Tris buffer (pH 8.3) and 0.5 ml. of an aqueous extract of an acetone powder of beef heart mitochondria. After this preincubation, which insured complete reduction of DPN, 0.5 ml. of a freshly prepared solution of oxaloglycolate containing 6 μmoles of the partly neutralized acid was added. The pH of the final reaction mixture was 7.0. The enzyme mixture was again incubated for 10 minutes, then deproteinized with 2 ml. of 10 per cent $HClO_4$, and the filtrate analyzed for keto acids and tartrate. The results were compared with those of an identical enzyme reaction mixture which was deproteinized at zero time; *i.e.*, just

at the moment oxaloglycolate was added. During 10 minutes incubation, 0.344 μmole oxaloglycolate was reduced. The rate of the reverse reaction is of the same order of magnitude as the oxidation of tartrate to oxaloglycolate (compare with Table II). Only traces of hydroxypyruvate (0.006 μmole) and no glyoxylate at all were found. Neither alcohol dehydrogenase nor the extract of mitochondria reduced glyoxylate to glycolate in the presence of DPNH. The color reaction of tartrate could not be quantitatively evaluated in this range; however, the appearance of the red metavanadate complex (absorption maximum at 505 mμ) of one of the optically active enantiomorphs clearly indicated that tartrate was actually formed.

Methods

Chemical Preparations—Dihydroxyfumaric acid was made by a modified procedure of Fenton (16) as described by Nef (17). The method recently published by Hartree (18) is essentially the same as that of Nef and does not appear to offer any advantages over that of the older paper. Hydroxypyruvic acid was prepared from chloropyruvic acid as described by Sprinson and Chargaff (19). Mesoxalic and diketosuccinic acids were obtained from the Aldrich Chemical Company, Inc., both as free acids and as the sodium salt. The properties of the acids agreed closely with those described by Fenton (20). Glyoxylic acid was obtained by the reduction of oxalic acid as described by Weinhouse and Friedmann (21). The 2,4-dinitrophenylhydrazine derivatives were prepared by dissolving the keto acids in an excess of the reagent (in 2 N HCl). After standing for 2 hours at room temperature, the mixture was kept at 4° overnight, and the crystals were removed by filtration, washed with 2 N HCl, and dried. Under such conditions the 2,4-dinitrophenylhydrazone of oxaloglycolic (from dihydroxyfumaric) and glyoxylic acids was obtained. Dihydroxyfumaric acid was quantitatively recovered as the 2,4-dinitrophenylhydrazone of the keto form from an aqueous solution. Diketosuccinic and hydroxypyruvic acids under these conditions yielded quantitatively the bis-2,4-dinitrophenylhydrazone (osazone).

Analytical Methods—Each derivative was purified by descending chromatography on Whatman No. 1 filter paper, which was previously impregnated with 0.1 M potassium phosphate buffer of pH 7.3 and dried. The solvent was ethanol (83 per cent) and H_2O (17 per cent). The chamber was equilibrated with 95 per cent ethanol. For the quantitative analyses of the keto acid derivatives the 2,4-dinitrophenylhydrazones were eluted from the paper and determined spectrophotometrically (12).

The *reduction of 2,4-dinitrophenylhydrazones to amino acids* was carried out as follows: Approximately 3 to 5 mg. of the 2,4-dinitrophenylhydrazine

derivative were dissolved in 30 ml. of glacial acetic acid, 1 mg. of platinum oxide catalyst was added, and the mixture vigorously shaken in H_2 atmosphere for 3 to 6 hours at room temperature. After the uptake of H_2 ceased, the solution was filtered and dried *in vacuo*. The residue was taken up in a small volume (0.05 to 0.1 ml.) of glacial acetic acid and quantitatively applied to a sheet of Whatman No. 1 filter paper and chromatographed (descending technique) (13). The amino acid was located by the ninhydrin reagent on a small strip of the total chromatogram, and the rest was purified by reextraction and further chromatography.

DISCUSSION

The results of experiments obtained with the tartrate oxidizing enzyme system suggest a definite pattern of consecutive enzymatic reactions. There is little doubt that the first step consists of the dehydrogenation of tartrate to oxaloglycolate. This type of dehydrogenation reminds us of the reaction catalyzed by malic dehydrogenase. Malic dehydrogenase is specific for the naturally occurring L(−)-malate (22) and does not act on dihydroxyfumarate, while the "tartrate dehydrogenase" acts on D(−)- and *meso*-tartaric acids. Malic dehydrogenase can react with both di- and triphosphopyridine nucleotides (23) as coenzymes; on the other hand, tartaric acid dehydrogenation occurs only in the presence of DPN. Various preparations of purified malic dehydrogenase of the type described by Straub (24) oxidize to a small and varying degree D(−)- and *meso*-tartrate; however, these preparations contain other enzymes (transaminases) and cannot be considered suitable for experiments which are to decide whether or not malic dehydrogenase acts on both substrates or, alternatively, whether different dehydrogenases are involved.

The main pathway of the enzymatic degradation of oxaloglycolic acid leads to the formation of glyoxylic acid. The formation of diketosuccinate was experimentally demonstrated, but we do not consider this reaction to be on the main path to glyoxylate. It should be mentioned that besides Banga, Szent-Györgyi, and Philippot (3, 4) who worked with plant extracts, Swedin and Theorell (25, 26) and more recently Chance (27) also studied the enzymatic oxidation of dihydroxyfumaric acid by purified horseradish peroxidase. This reaction is catalyzed by a Mn^{++}-activated peroxidase-peroxide complex (27) and occurs under different conditions (pH 4.7) from the oxidation of tartaric acid (pH 8.3). According to Chance (27) the oxidation of dihydroxyfumaric acid by the peroxidase-peroxide complex requires Mn^{++}, while the formation of glyoxylate from tartrate goes equally well with Mg^{++}, which was used exclusively in all our experiments.

While diketosuccinate decomposes non-enzymatically to CO_2 and tar-

tronate, glyoxylate appears only in the presence of the enzyme system, DPN, and tartrate as substrate.   Dihydroxyfumarate is enzymatically reduced to tartrate (in the presence of continually regenerated DPNH), but it does not yield glyoxylate.   This observation suggests that the precursor of glyoxylate is not the dienol but the keto form of dihydroxyfumarate, *i.e.*, oxaloglycolate.   This question could not be clarified by the identification of oxaloglycolate as its 2,4-dinitrophenylhydrazone, since both the synthetic dienol and the enzymatically formed product yielded the same derivative upon prolonged (24 hours) incubation with the carbonyl reagent. On the other hand, it is known that dihydroxyfumarate is a relatively

I=MESO-TARTRATE   IV=HYDROXYPYRUVATE
II=OXALOGLYCOLATE   V=DIKETOSUCCINATE
III=GLYOXYLATE   VI=TARTRONATE
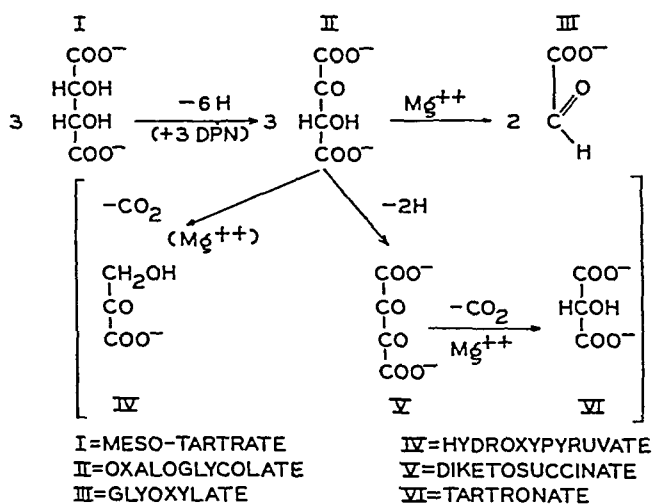
FIG. 3

stable dienol (18) and thus may not be acted upon by an enzyme which is specific for the keto form.   It is suggested that glyoxylate is formed from oxaloglycolate (which is the primary product of the dehydrogenation of tartrate) by an intramolecular transfer of H, followed by cleavage between C_2 and C_3.

Alternative possibilities of glyoxylate formation via the oxidative decarboxylation of hydroxypyruvate or tartronate were ruled out by experiments which showed that under given conditions (during tartrate oxidation) both added hydroxypyruvate and tartronate were inert and yielded no glyoxylate.   Concomitantly with this major pathway of glyoxylate formation, a small but consistent decarboxylation of oxaloglycolate to hydroxypyruvate occurs.   It is not certain that this reaction is catalyzed by an enzyme.   The summary of major reactions is schematically outlined in Fig. 3, which merely represents a qualitative pathway.

It is hardly possible at present to predict whether or not the sequence of reactions outlined in this paper represents a major pathway of metabolism. The ubiquitous appearance of the enzyme reactions may indicate this. On the other hand, an interesting possibility may be suggested, that transaminations of some of the keto acids formed from tartrate result in amino acids which, in small amounts, could serve as physiological regulators of cell metabolism, as antimetabolites. Such examples have been described; e.g., diaminopropionic acid is a constituent of the antibiotic viomycin (28), and hydroxyaspartic acid as well as diaminosuccinic acid is known to inhibit the growth of *Escherichia coli* (29). These possibilities in animal, plant, and microbial metabolism as well as the characterization of the enzyme components of the tartrate oxidizing enzyme system are now under investigation.

SUMMARY

1. Suspensions of mitochondria prepared from various tissues of rat and beef oxidize $D(-)$- and *meso*-tartrate. The enzyme system was brought into solution by extraction. The reaction required DPN; added Mg^{++} increased O_2 uptake and CO_2 evolution.

2. The products of the enzymatic oxidation were isolated and identified as (a) oxaloglycolate, which yields (b) glyoxylate. The formation of (c) diketosuccinate, (d) hydroxypyruvate, and (e) tartronate was also established.

3. The reduction of oxaloglycolate to tartrate was demonstrated. The sequence of reactions and their possible rôle in cell metabolism are discussed.

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PLANT PHOSPHOLIPASE D

I. STUDIES ON COTTONSEED AND CABBAGE PHOSPHOLIPASE D[*]

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Phospholipases are of current interest in two rather distinct lines of research: the general interest in phospholipide metabolism, and the special effects of such enzymes on terminal oxidase systems. The present paper deals with certain descriptive observations on the relatively little studied phospholipase D[1] obtained from two plant sources. A subsequent paper is concerned with its inactivating effect on succinic oxidase.

Phospholipase D is a widely distributed enzyme, occurring in such unrelated plants as cabbage (5), Hevea latex (6), soy beans, maple trees (7), etc. The phospholipide of seeds disappears during germination, reappearing as water-soluble choline and other compounds (7), thus suggesting a general rôle for the enzyme in the utilization of reserve phospholipide.

Nearly all of the work on phospholipase D has been done by measuring only the choline released from lecithin. However, Rose (8) found that the cabbage enzyme released either serine or ethanolamine from "cephalin." Kates (9, 10) has recently shown that in certain leaves, notably spinach and cabbage, the enzyme is confined to the chloroplast fraction, but no soluble preparations of phospholipase D have been reported. It now appears, however, that the enzyme from a new source, cottonseed, can exist in an apparently soluble form. It attacks phosphatidyl ethanolamine as well as lecithin. The principal distinguishing properties for the cottonseed enzyme are its apparent solubility in water and its lack of activation by diethyl ether.

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[1] Phospholipase D hydrolyzes phospholipide to release free nitrogen base and phosphatidic acid. Since these enzymes also cleave substrates other than lecithin, they are termed phospholipases. Some authors refer to phospholipase D as phospholipase C or lecithinase C, following the prophecies of Contardi and Ercoli (1); we and others (2) follow the names assigned as the enzymes were discovered: lecithinase C (Clostridium welchii α-toxin) was named in 1941 (3) before phospholipase D was discovered (4).

Materials and Methods

Enzymatic Assay—The assay for cephalin hydrolysis was adapted from Rose (8). In many instances it was convenient to use smaller quantities of enzyme. This was accomplished by conducting the reaction in the microdiffusion apparatus of Kirk and Tompkins (11). 1 ml. of reaction mixture usually contained 4 mg. of enzyme (dry weight), water, and 40 mg. of substrate emulsified in phosphate buffer to give a final concentration of 0.1 M phosphate. After incubation, the reaction was stopped with 0.4 ml. of saturated potassium metaborate (12). 0.1 ml. of 2 M periodic acid (H_5IO_6) was placed in the larger bulb of the vessel (not in contact with the digest), 0.05 ml. of 20 per cent sulfuric acid was placed in the spoon, the cell evacuated, and the periodic acid then mixed with the digest. The ammonia was allowed to diffuse for 2 hours at room temperature, after which the sulfuric acid was made up to 2.00 ml. and nesslerized (13). A Beckman model B spectrophotometer at 490 mμ was used to measure the color developed. Controls were run with all assays either by using boiled enzyme or by adding the enzyme after the addition of the metaborate. Only free ethanolamine and serine[2] are measured by the method. Release of choline from lecithin was measured separately by the aqueous Reinecke salt method of Snell and Snell (14).

Purified soy bean "lecithin RG" was used as a substrate throughout. This is a mixture of phospholipides having an approximate percentile analysis as follows:[3] cephalin, 29.2; lecithin, 29.2; inositol phosphatides, 31.4; glycerides, 4.0; sugars, glucosides, 5.2; insoluble materials, moisture, 1.0.

Chemical Methods—Inorganic phosphate was determined by the method of Allen (16). Turbidity in the solutions was avoided by coagulation of lecithin RG with $HClO_4$, followed by centrifugation. Enzymatic digestions were carried out in 0.1 M acetate buffer at pH 5.9 when phosphate was to be measured subsequently.

Calcium phosphate gel was prepared according to Kunitz (17).

Free fatty acids were determined by the method of Fairbairn (18).

Chromatography—Whatman No. 1 paper "for chromatography" was employed throughout. Lutidine-ethanol and phenol-water developing solvents are described by Block (19). Butanol-acetic acid (Solvent I) was made by shaking together 49 ml. of *n*-butanol, 49 ml. of distilled H_2O,

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[2] Threonine would also be measured, but this is not known to occur in phospholipides. The results are reported as ethanolamine N, since no serine is liberated by the enzyme, as shown by paper chromatograms.

[3] Obtained through the courtesy of Mr. John Lathe of The Glidden Company. The analytical data were also furnished by The Glidden Company and are in quite good agreement with those of Scholfield *et al.* (15).

and 2 ml. of glacial acetic acid, then using the upper phase. Butanol-acetic acid (Solvent II) consisted of 4 volumes of n-butanol, 1 volume of glacial acetic acid, and 1 volume of water. 0.2 per cent ninhydrin in water-saturated n-butanol was used as a color reagent.

Digests for chromatography were prepared in a manner designed to concentrate the end-products. For example, 10 mg. of the cottonseed enzyme were incubated for 6 hours with 100 mg. of lecithin RG in the presence of 0.4 M NaCl and 0.1 M phosphate buffer at pH 5.9. (The total volume was 2.5 ml.) Boiled enzyme was used for the control. The digest was acidified, extracted with ether, and 2 ml. of the aqueous phase were evaporated to 0.2 ml. 5 μl. of the concentrate were then applied to the paper. The digests contained appreciable amounts of NaCl, but this did not interfere in the preparation of chromatograms suitable for showing ethanolamine and serine.

<div align="center">RESULTS AND DISCUSSION</div>

<div align="center">*Enzyme Preparations*</div>

Cabbage Enzyme—The enzyme from cabbage leaves was prepared by homogenizing fresh leaves, from which the center ribs had been removed, with an equal weight of water, saturating the press juice therefrom with NaCl, and dialyzing the resulting precipitate against distilled water as in Step B below. The suspension was then lyophilized to yield a stable pale green powder.

Cottonseed Enzyme. Step A; Crude Extract—Cottonseed meal[4] was extracted with hexane and air-dried. The meal was next disintegrated with water (8 to 12 ml. per gm.) in a colloid mill, and the pH was adjusted to 8.2 to 8.4 with NaOH. After standing in the cold 2 hours, the mixture was centrifuged at 800 × g to remove the larger particles.

Step B; NaCl Precipitate—The crude extract was saturated with NaCl and allowed to stand 4 hours in the cold. The precipitate was collected by 40 minutes centrifugation at 26,000 × g (average centrifugal force) and dialyzed overnight against 400 volumes of cold distilled water. The precipitate does not dissolve appreciably.

Alternative Step B; Elution from Calcium Phosphate Gel—The crude extract from cottonseed was treated with twice its volume of calcium phosphate gel at pH 6.1, and the gel eluted with 0.07 M phosphate buffer at pH 8.2. The eluate was dialyzed overnight against 50 volumes of cold distilled water and lyophilized. The preparation does not precipitate on dialysis.

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[4] Cottonseed "meats" specially prepared without heating were obtained through the courtesy of Dr. Willy Lange of The Procter and Gamble Company.

Step C; Second Extract—The contents of the dialysis bag (from Step B) were extracted with distilled water at a slightly alkaline pH and centrifuged 20 minutes at 26,360 × *g*. The clear supernatant liquid was adjusted to pH 8.0 and lyophilized. While wet, the enzyme was unstable, but the dry preparations retained their activity completely in cold storage for at least 4 months.

The degree of enzyme purification achieved is summarized in Table I. All values are averages of at least two determinations.

TABLE I

Steps in Purification of Cottonseed Phospholipase D

Enzyme preparation	Specific activity	Yield
	units per mg. substance	*units* per gm. meal
Defatted meal†	0.22	220
Step A; crude extract		344
" B; NaCl ppt., lyophilized	5.9‡	384
" C; 2nd extract	19.0	357
Alternative Step B; calcium phosphate gel	9.1	157

* For brevity, the activity has been expressed in units defined as follows: 1 unit corresponds to the release of 1 γ of ethanolamine nitrogen in 15 hours at 25° in 0.10 M phosphate buffer at pH 5.9. The system contained 60 mg. of enzyme preparation and 200 mg. of lecithin RG in a total volume of 4.0 ml.

† Prepared by mixing the meal with 0.10 M phosphate, pH 5.9. Increasing yields might be attributed to a pH difference between this and the other preparations, although the presence of an inhibitor is not excluded.

‡ The usual preparation from cabbage leaves corresponding to this step had a specific activity of 5.0 units per mg.

Cottonseed phospholipase D has thus been obtained in an aqueous fraction which is not sedimented by 20 minutes centrifugation at 26,360 × *g*, in contrast to cabbage phospholipase D which is in the chloroplast fraction (10). Since various authors give values from 10,000 to 20,000 × *g* as sufficient for the removal of plastids and mitochondria (20, 21), it is felt that the cottonseed phospholipase D is soluble, or at least a very small particle.

By way of a further distinction between the two enzymes, the enzyme from cabbage leaves has been found to be potently activated by saturation of the aqueous medium with diethyl ether (10). Under corresponding conditions, we found the cottonseed enzyme to exhibit only 70 per cent of its normal activity. It is plausible that the particulate nature of the cabbage preparation is responsible for this difference in properties.

pH Optimum—The pH observed as optimal was essentially the same with preparations from both cottonseed and cabbage, as shown in Fig. 1.

Calibration of Assay—The relation between the ethanolamine liberated at pH 5.9 and the amount of enzyme present is shown in Fig. 2; that between observed activity and amount of substrate in Fig. 3. These findings for the release of ethanolamine are similar to the results of Kates (10)
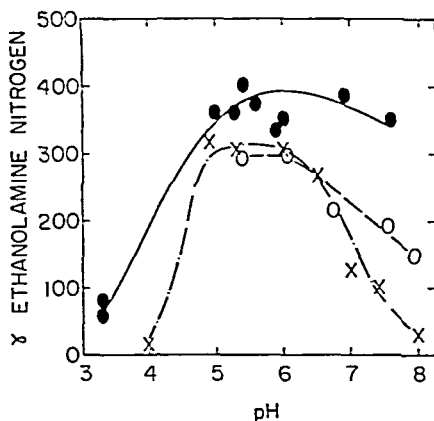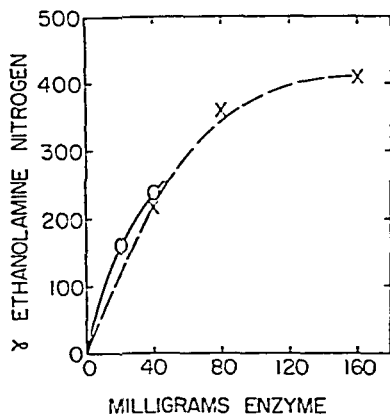
FIG. 1   FIG. 2

FIG. 1. pH dependence of phospholipase D activity. One preparation from cabbage leaves (×), and two preparations (Step B, lyophilized) from cottonseed (⊙, ●). The ethanolamine measured was that released in 15 hours at 25° by 60 mg. of enzyme preparation from 200 mg. of substrate in 0.07 to 0.10 M phosphate buffer. Total volume 4.0 ml.

FIG. 2. Dependence of activity on concentration of enzyme. Liberation of ethanolamine by cottonseed preparation (Step C) (⊙) was measured after 2 hours at 30° (400 mg. of lecithin RG, total volume 10.0 ml.); by cabbage leaf preparation (×) after 15 hours at 25° (150 mg. of lecithin RG, total volume 4.0 ml.). 0.10 M phosphate buffer at pH 5.9 was employed.

for the release of choline by the cabbage enzyme. With the Lineweaver-Burk equation (22), an apparent Michaelis constant of 2.1×10^{-2} M may be calculated for cabbage phospholipase D by using the data of Fig. 3.

Stability Studies—The thermal stability of the cottonseed enzyme was determined by assays after 5 minute exposures to various temperature levels in 0.07 M phosphate buffer at pH 5.9. The results (Fig. 4) indicate that the enzyme possesses no unusual heat stability.[5] The thermal stability of the cabbage enzyme has been extensively studied elsewhere (5, 10); it was not studied on these preparations, but it was noted that their enzymatic activity was destroyed by brief boiling.

The pH range of optimal stability of the cottonseed enzyme was tested by exposure at room temperature for 4.5 hours. It proved to be rather

[5] This observation does not necessarily apply to the stability in the whole seed, where lipides are high and water relatively low. The question has been of some industrial importance.

broad, there being less than 10 per cent destruction of the enzyme between pH 6.40 and 8.75. Despite its wide range, the pH of optimal stability does not coincide with the pH of optimal activity.

Specificity—Compared with the amount of nitrogenous base liberated by our cottonseed preparations, the liberation of inorganic phosphate was quite small. The concomitant release of choline, ethanolamine, and inorganic phosphate is reported in Table II. The preparations are thus
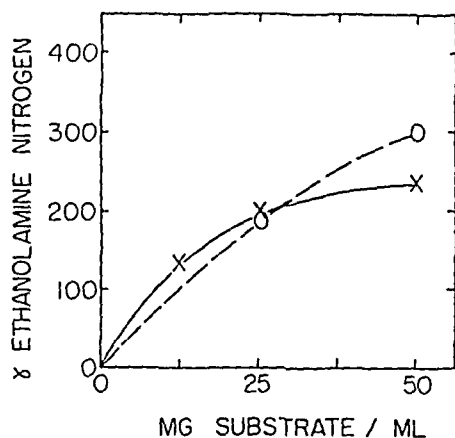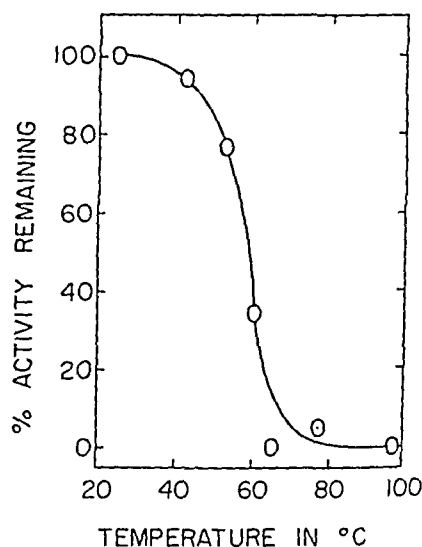
Fig. 3   Fig. 4

Fig. 3. Dependence of activity on concentration of lecithin RG with cottonseed preparation (⊙) and cabbage leaf preparation (×). The cottonseed preparation (20 mg., Step C) was incubated for 2 hours at 30° and contained 0.10 M phosphate, 0.5 M NaCl, in a volume of 4.0 ml. at pH 5.9. The cabbage enzyme (equivalent of 33 gm. of leaves) was incubated for 9 hours at 25° in 0.07 M phosphate, in a volume of 4.0 ml. at pH 5.9.

Fig. 4. Thermal inactivation of cottonseed phospholipase D. Per cent activity remaining after 5 minute exposures at the indicated temperatures. Ethanolamine released in 15 hours at 25° from 200 mg. of lecithin RG by 60 mg. of cottonseed phospholipase D (Step B), in 0.07 M phosphate, in a volume of 4.0 ml. at pH 5.9.

shown to give an effect typical of phospholipase D, with very little subsidiary phosphatase action.[6] In like manner, there is little free fatty acid released (Table II), indicating that the preparation is relatively free from lipase or phospholipase A action.

An interesting but still open question about phospholipase D concerns its substrates among the phospholipides. The cabbage and carrot prep-

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[6] For similar data on cabbage preparations, see Hanahan and Chaikoff (5) and Kates (10), where the results are quite similar to those reported here for the cottonseed enzyme.

arations used by Hanahan and Chaikoff (4, 5) liberated choline from a mixture of phospholipides; similar (but not identical) preparations were observed to liberate either ethanolamine or serine by a method in which both are measured ((8), also this paper). It follows from Table II that the enzyme from cottonseed hydrolyzes both lecithin and phosphatidyl ethanolamine. The cabbage enzyme also acts on both of these substrates.

Chromatograms prepared from the substrate after acid hydrolysis (6 hours boiling in 2.5 N HCl) indicated that little or no serine was present (Fig. 5). The existence of phosphatidyl serine in "soy bean lecithin" has

TABLE II

Action of Cottonseed Phospholipase D on Soy Bean Lecithin RG

	Release of phospholipide hydrolysis products			
	Experiment A		Experiment B	
	μmoles	Moles released / Moles total N released	μmoles	Moles released / Moles total N released
Inorganic P	0.26	0.05		
Free fatty acid*........			1.4†	0.13
Ethanolamine N	1.7	0.35	3.3	0.31
Choline N	3.2	0.65	7.4	0.69

Experiment A, 4 mg. of cottonseed phospholipase D (Step C) incubated 2 hours at 30° with 40 mg. of lecithin RG in 0.10 M acetate buffer at pH 5.9. Total volume 1.0 ml. *Experiment B*, same as Experiment A except in 0.10 M phosphate buffer, pH 5.9, and in the presence of 0.4 M NaCl as an activator. All values are an average of at least two determinations.

* Calculated as linoleic acid.

† The experimental results were 5 times larger than shown. All ingredients were increased 5-fold because of the limits of the method.

been postulated on occasion, but seems to be dubious. Scholfield's (15) separation of soy bean phospholipides did not rule out the presence of serine, because his method of analysis did not distinguish between ethanolamine and serine. In order to identify the nitrogenous base released by the enzymes, soy bean lecithin RG, the usual substrate, was digested with enzyme preparations from both sources. The resulting material was then examined by paper chromatography. Paper chromatograms developed with a variety of solvents always showed spots corresponding to ethanolamine, but never to serine, in the digests of both cottonseed and cabbage phospholipase D. These were developed with several solvents as one-dimensional chromatograms, and also as two-dimensional chromatograms with n-butanol-acetic acid (Solvent I), fol-

lowed by either of the other solvents described. However, cleavage of phosphatidyl serine cannot be eliminated as a property of the enzymes, but it is not a factor in this case.
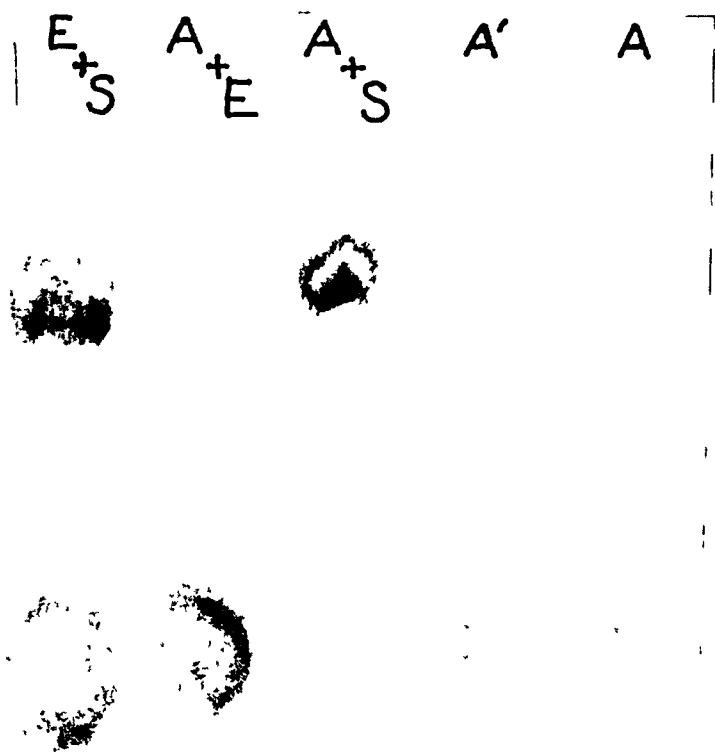
Fig. 5. Chromatogram of an acid hydrolysate of lecithin RG. *E*, known ethanolamine; *S*, known DL-serine; *A*, 5 μl. of hydrolysate; *A'*, 10 μl. of hydrolysate. Developed with *n*-butanol-acetic acid-water (4:1:1); sprayed with ninhydrin. The lowest (third) spot on *E* + *S* appears to be a contaminant of redistilled ethanolamine.

Effect of NaCl—A curious effect of NaCl was observed on cottonseed phospholipase D. With the routine preparation, the presence of 0.4 M NaCl gave 60 to 75 per cent activation and tended to give greater activation with increasing time of digestion. On the other hand, with a preparation made with calcium phosphate gel (alternative Step B), 0.4 M NaCl gave 118 per cent activation during a 15 hour incubation (Table III), but during a 2 hour incubation it inhibited the phospholipase D, the degree

of inhibition varying with the salt concentration. This reversal of effect might be due to some protective action of the salt during the longer in-

TABLE III

Effect of NaCl on Cottonseed Phospholipase D

Enzyme preparation	Length of incubation	NaCl	Per cent change in activity
	hrs.	M	
Step C	2	0.5	+60
	2	0.4	+64
	3	0.4	+74
Alternative Step B	2	0.001	0
	2	0.01	0
	2	0.10	−39
	2	0.5	−31
	2	1.0	0
	15	0.4	+118

The complete system contained 20 mg. of enzyme, 200 mg. of lecithin RG, and NaCl in 0.10 M phosphate, pH 5.9, in 4.0 ml. total volume. The incubation temperature was 30° except in the 15 hour assay, which was at 25°.
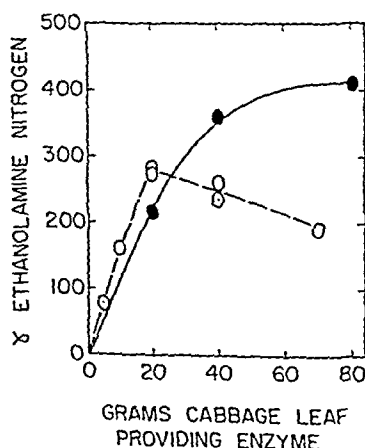
FIG. 6. Relation of activity to amount of cabbage enzyme used. Ethanolamine released in 15 hours at 25° from 150 mg. of lecithin RG in 0.07 M phosphate buffer at pH 5.9. Total volume 4.0 ml. Undialyzed NaCl precipitate (⊙); dialyzed NaCl precipitate (●). For details see the text.

cubation. Our preparations of cabbage phospholipase D were also activated by NaCl, 1.0 M NaCl giving 22 per cent activation during a 15 hour incubation, but inhibition by NaCl was not observed under the conditions employed.

Naturally Occurring Inhibitor—The enzyme in cabbage leaves appears to be accompanied by a material inhibitory thereto. In the case of the precipitate obtained from cabbage leaf juice with NaCl, large doses of

TABLE IV

Effect of Inhibitor from Cabbage Leaves

Enzyme preparation	Inhibitor			Per cent change in activity
	Corresponding weight of cabbage leaves	Heat treatment	Weight	
	gm.		*mg.*	
12 mg. cabbage preparation	2.6	Boiled 2 min.		−62
	2.0			−72
	2.0		50	−34
	2.0		50	−45
	2.0	Boiled 3 min.	50	−24
	2.0	Ashed	4.4	+30
	2.0	"	4.4	+33
2 mg. cottonseed (Step C)	2.0		50	+15
	2.0		50	+29

The complete system contained enzyme, inhibitor, and 40 mg. of lecithin RG in 0.10 M phosphate buffer, pH 5.9, in a total volume of 1.0 ml. The ethanolamine released was measured after 15 hours incubation at 25°.

TABLE V

Comparison of Phospholipase D from Cottonseed and Cabbage

	Cottonseed	Cabbage*
Physical state	Withstands 26,360 × *g*	Particulate (10)
pH optimum	pH 5.0–6.0	pH 5.0–6.0
Digestion of lecithin	Occurs	Occurs (5, 10)
" " phosphatidyl ethanolamine	"	"
Release of inorganic P and free fatty acids	Low	Low (5)
pH stability	pH 6.4–8.75	
Thermal stability	Destroyed at 70°	Destroyed by boiling, but see (5) for contrary finding
Activation by NaCl	Occurs	Occurs
Inhibitor in cabbage	Not inhibited	Inhibited
Activation by diethyl ether	" activated	Activated (10)

* The figures in parentheses refer to the bibliography.

material prior to dialysis had less effect than smaller doses, as shown in Fig. 6. After dialysis this effect disappeared, and the inhibitory material[7] could be recovered from the diffusate. Thus when cabbage press juice was brought to pH 2 to 3 and dialyzed against distilled water, which was then evaporated to dryness at low temperature, a solid was obtained. When dissolved in water and adjusted to pH 5 to 6, the solid exhibited inhibitory power, as shown in Table IV.

The inhibitory material is slowly destroyed by boiling and completely destroyed by ashing; the inorganic ash produces instead a small activation (Table IV). It is plausible that the inhibitory material from cabbage contains activating salts and an organic inhibitor which overcomes them. It should be noted that the inhibitory effects seen are small, considering the amounts necessary to produce inhibition.

The material from cabbage did not inhibit the cottonseed enzyme (Table IV). The cottonseed activation may well be due to the salts present in the "inhibitory" material.

The discussion of a purely descriptive study must necessarily concern itself with details. The similarity of phospholipase D in cottonseed to that in cabbage leaves is striking. The principal differences observed between the behavior of the enzyme in the two preparations can well be attributed to particle size (or solubility). A comparison of these preparations is presented in Table V. Whether the similarity bespeaks a common function must obviously await further study.

SUMMARY

An enzyme which releases ethanolamine and choline from phospholipides is reported in cottonseed. It is obtained as a stable dry product with a specific activity against phosphatidyl ethanolamine 86 times that of defatted cottonseed meal. An aqueous solution of the enzyme withstands a centrifugal force of $26{,}360 \times g$ for 20 minutes. This is the first report of an apparently soluble phospholipase D.

The similar enzyme from cabbage is shown to release ethanolamine from phospholipide. An inhibitor in cabbage juice is described.

The pH optimum, thermal stability, and the effects of diethyl ether and of NaCl on the cottonseed enzyme are described and compared to those of the cabbage enzyme.

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THE METABOLISM OF THE ORGANIC ACIDS OF TOBACCO LEAVES

X. EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF FUMARATE AND MALEATE

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A preliminary experiment some years ago showed that fumaric acid supplied to tobacco leaves by the leaf culture technique readily enters into the organic acid metabolism (1). Both malic and citric acids increased markedly, and the group of undetermined acids, in which any excess of fumaric acid present would have been included, increased to only a small extent. The effects were in general quite similar to those observed when succinic acid was administered in the same way. The development of chromatographic methods for the determination of organic acids in plant extracts (2) and the improved sampling methods now employed have recently made it possible to reinvestigate the behavior of fumaric acid in greater detail under better experimental conditions. In addition, the study has been extended to a consideration of the influence of the isomeric substance maleic acid upon the organic acid metabolism of tobacco leaves.

No previous test of the effect of the administration of maleic acid to leaves has come to our attention although there are a few records of experiments with this substance in other connections. For example, Greulach (3) observed that young bean or sunflower plants dipped for a short time into 0.015 M solutions of sodium maleate subsequently exhibited an enhanced rate of growth. Lundegärdh (4) found that the respiration of young wheat roots is stimulated when they are immersed in a 0.002 M solution of potassium maleate at pH 6.8, the stimulation being somewhat diminished in the presence of cyanide. Thimann and Bonner (5) have noted that the inhibition of the growth of *Avena* coleoptiles by iodoacetate is relieved by a number of organic acids, maleate being one of the moderately effective ones, and McRae, Foster, and Bonner (6) have made use of 0.0025 M potassium maleate buffer solutions at pH 4.5 in their studies of the effects of auxins on the growth of coleoptiles. Such observations as these indicate that dilute solutions of salts of maleic acid are not toxic to plant tissues and that the acid may have definite effects upon the general metabolism; in fact, Sacks and Jensen (7) have presented evidence for the

conversion of maleic acid into malic acid by an enzyme preparation from maize kernels.

On the other hand, maleic acid is a recognized inhibitor of the activity of certain enzymes, presumably because of its capacity to react with thiol groups under physiological conditions (8). However, enzymes differ widely in their sensitivity to maleic acid, the succinic acid oxidase system of pigeon brain tissue, for example, requiring a far higher concentration for significant inhibition than pyruvic acid oxidase from the same source (9). Furthermore, ample time must be allowed for the interaction with maleic acid to take place if inhibition is to become pronounced.

The present experiments have shown that fumaric acid is taken up by tobacco leaves from 0.2 M solutions at pH 5 or 6 in somewhat smaller amounts than succinic acid under similar conditions. Maleic acid is taken up in much larger amounts. As was found in the earlier test, fumaric acid seems to behave, with respect to its influence upon the metabolism of malic and citric acids, in a manner similar to that of succinic acid, for it was extensively metabolized and the production of citric acid was greatly stimulated. Maleic acid, however, although it was also metabolized to an appreciable extent, depressed the formation of citric acid. In the presence of either fumaric or maleic acid, the extent to which malic acid was used up in the metabolism was greatly diminished. The evidence indicates, furthermore, that maleic acid made a significant contribution to the respiratory loss of organic solids from the system.

EXPERIMENTAL

Samples of twenty leaves each, ten in number, were collected by the statistical method (10) on September 22, 1953, 55 days after the seedlings had been set out in the greenhouse. The species was *Nicotiana tabacum* var. Connecticut shade-grown, although, in the preliminary experiment (1), *Nicotiana rustica* had been used. The coefficient of variation of the fresh weight of the samples was 1.6 per cent, that of the total nitrogen 2.0 per cent. One sample was dried at once for analysis, three were cultured for 24 hours, respectively, in water, in 0.2 M potassium fumarate at pH 5, and in 0.2 M potassium fumarate at pH 6, and three for 48 hours under the same conditions. In addition, three samples were cultured for 48 hours, respectively, in 0.2 M potassium maleate at pH 5, in 0.2 M potassium maleate at pH 6, and in 0.2 M potassium succinate at pH 5, the last to serve as a basis of comparison. The dark room was held at 24° and 50 per cent relative humidity during the culture period, and all samples were dried at 80° in a ventilated oven and subsequently equilibrated with air at 24° and 50 per cent relative humidity in preparation for analysis.

The leaves cultured in water, in fumarate, and in succinate maintained

their turgidity and increased somewhat in fresh weight (Table I, Line 1); all appeared to have been substantially unharmed. The leaves cultured in maleate, however, soon became flaccid, and in 48 hours had lost nearly one-half of their initial fresh weight; they became extremely thin and fragile and only the midribs retained any turgidity. The physical behavior was quite similar to that of tobacco leaves cultured in oxalate (11).

The analytical data were obtained by methods that have been described in recent bulletins from this Station (12, 2), and the results for the samples cultured for 48 hours are collected in Table I. The data for the samples cultured for 24 hours are omitted since they merely showed that the various changes in composition, although usually more extensive during the first 24 hour period than in the second, continued throughout.

The increases of the ash (Line 3) in the leaves cultured in fumarate did not differ greatly from those in the leaves cultured in maleate, but, owing to differences in uptake of the acid, the increases in the corrected organic solids (Line 5) were widely different. The uptake of each of the three acids (Line 7) was computed from the respective increase in the alkalinity of the ash (Line 6) with the use of factors calculated from the dissociation constants of each acid. Since approximately 90 per cent of fumaric acid is neutralized at pH 5 and 99 per cent at pH 6, the data for fumaric acid are probably reliable. Maleic acid, however, is only approximately 53 and 69 per cent neutralized at these two acidities and succinic acid is only 54 per cent neutralized at pH 5; the accuracy of the data derived with use of these quantities is accordingly somewhat less certain, but the figures nevertheless provide reasonably close estimates.

Malic acid (Line 10) diminished and citric acid (Line 11) increased in the control leaves cultured in water in the manner to be anticipated from previous studies, although more extensively than is sometimes seen. This particular lot of tobacco leaves was apparently characterized by an unusually vigorous organic acid metabolism. When cultured in fumarate, the loss of malic acid from the leaves was only about one-quarter as great as in the water control, but the increase in citric acid was nearly doubled. Culture in succinate brought about an increase both in malic acid and in citric acid, but the increase in citric acid, as compared with that of the water control, was only a little more than half as great as the increase stimulated by culture in fumarate. It is clear that both succinic and fumaric acids share the capacity to stimulate the formation of citric acid in tobacco leaves. The difference between them appears in part at least to be referable to the relative amounts of these acids taken up by the leaves under the experimental conditions (Line 7); at pH 5, only about three-quarters as much fumaric acid was taken up as succinic acid, and, accordingly, less

TABLE I

Effect of Culture in 0.2 M Solutions of Potassium Fumarate, Maleate, and Succinate upon Composition of Excised Tobacco Leaves

Data expressed in gm. or milliequivalents per kilo of initial fresh weight of leaves.

Line No.		Control before culture	Changes during culture in darkness for 48 hrs.					
			Water	Potassium fumarate		Potassium maleate		Potassium succinate
				pH 5	pH 6	pH 5	pH 6	pH 5
1	Final fresh weight per kilo of initial fresh weight, *gm.*	1000	1167	1077	1054	511	531	1129
2	pH of extract of dry tissue	5.22	+0.08	+0.09	+0.16	+0.16	+0.63	+0.17
3	Inorganic solids, *gm.*	20.6	−0.1	+12.4	+12.6	+11.5	+16.2	+9.3
4	Organic solids, *gm.*	85.0	−5.4	−1.8	−2.7	+5.0	+4.2	−0.9
5	" solids, corrected for CO_2 of ash, *gm.*	93.1	−5.5	+1.6	+0.7	+8.4	+9.1	+1.8
6	Alkalinity of ash, *m.eq.*	365	−6.0	+153	+156	+153	+223	+123
7	Uptake of acid, *m.eq.*			171	158	289	325	226
8	Uptake of acid, *gm.*			9.9	9.1	16.8	18.8	13.4
9	Total organic acids, *m.eq.*	283	−8.1	+165	+155	+258	+266	+139
10	Malic acid, *m.eq.*	198	−62.6	−17.0	−17.1	−18.8	−13.7	+38.3
11	Citric " "	22.7	+50.1	+96.8	+91.8	+21.7	+41.1	+74.1
12	Oxalic " "	32.8	+3.9	+3.9	+4.3	+1.1	+2.0	+0.2
13	Fumaric, maleic, or succinic acid,* *m.eq.*	0		+86.4	+76.2	+239	+205	+18
14	Unknown Acid A, *m.eq.*	18	+0.3	−7.2	−6.4	+9.5	+23	+2.5
15	Undetermined acids, *m.eq.*	13	0.1	+6.8	+6.1	+2.4	+8.3	+5.5
16	Protein N, *gm.*	3.07	−0.24	−0.44	−0.43	−0.16	−0.06	−0.38
17	Starch, *gm.*	2.07	−1.8	−1.9	−2.0	−1.5	−1.7	−2.0

* Neither fumaric nor maleic acid was detected in normal tobacco leaves by the present methods, but approximately 1 m.eq. per kilo of succinic acid is present. No fumaric acid was detected in the leaves cultured either in maleic or in succinic acid.

substrate was available for the presumed transformation into citric acid. As a result, some of the malic acid originally present in the leaves was drawn upon when fumaric acid was supplied. This did not occur when succinic acid was made available. As compared with the behavior of the water control, fumaric acid may, therefore, be said to have "spared" the utilization of malic acid, whereas succinic acid apparently made a direct contribution to the quantity of this substance present. This interpretation of the behavior of fumaric acid is rendered the more probable in the light of the early preliminary experiment (1) in which stimulation of the formation of both malic and citric acids occurred to about the same extent in leaves cultured either in fumarate or succinate at pH 5.5. Nevertheless, the interpretation is dependent upon the assumption that fumaric acid is transported out of the lumina of the vascular system of the leaf into the cells as readily as succinic acid appears to be.

Malic acid diminished in the leaves cultured in maleate at pH 5 to about the same extent as in the leaves cultured in fumarate at the same reaction, i.e. malic acid may seem again to have been "spared"; nevertheless, the formation of citric acid in the culture at pH 5 was less than one-half of that in the water control and was also appreciably diminished in the culture at pH 6. The general course of the organic acid metabolism must, therefore, have been fundamentally altered by the influx of this substance.

Oxalic acid was not affected notably; the changes were all small and near the limit of measurement. However, the increase of 3.9 m.eq. in the leaves cultured in water correlates with the manifestly high level of general metabolic activity in this lot of leaves. The similar increases in the leaves cultured in fumarate point to the same conclusion, and it was noted that the increases in the leaves cultured for 24 hours either in water or in fumarate were nearly as large. The increase of oxalic acid in the leaves cultured in succinate was, however, negligible. A hint is provided that the availability of a large amount of exogenous substrate in this case diminished the metabolic load upon the unknown substance which is normally converted into oxalic acid during the culture period. The increases of oxalic acid in the leaves cultured in maleic acid were intermediate between the effects upon the leaves in fumarate and succinate, and this is true also of the amounts of acquired acid which were metabolized during the culture period.

Line 13 shows the amounts of fumaric, maleic, and succinic acids found in the leaves cultured in these respective acids. Neither fumaric nor maleic acid could be detected in the control sample, and succinic acid was present in only a trace estimated to be 1 m.eq. per kilo. Furthermore, no fumaric acid could be detected in the samples cultured in either succinic

or maleic acid.[1]   Comparison of the data in Line 13 with the data for up-take in Line 7 shows that approximately one-half of the fumaric acid acquired by the leaves was metabolized at both pH 5 and 6.   Maleic acid was also moderately well utilized at both pH 5 and 6, but, since the uptake was much greater than that of fumaric acid, the proportions that entered into metabolic reactions were smaller.   Succinic acid was, as would be anticipated from previous results, nearly completely metabolized.

The Unknown Acid A in Line 14 represents a component which is eluted from the Dowex 1 analytical column together with citric acid.   Citric acid is determined by a pentabromoacetone method (13) in the pooled fractions, and the difference between the titratable acidity of these fractions and the citric acid found in them furnishes an estimate of the quantity of the unknown acid present.   It is a substance which occurs in tobacco leaves in amounts almost comparable with those of citric acid (cf. Table I, column 1) and appears to have been drawn upon during culture of the leaves in fumaric acid.   It increased materially in the leaves cultured in maleic acid, but did not change significantly in those cultured in succinic acid. Even though the accuracy with which this acid can be determined may not be great, since its stability under the conditions of the analytical method is not known, the presence of a substance which may play an active part in the organic acid metabolism is clearly indicated.

The remaining titratable acidity shown as undetermined acids[2] in Line

[1] The analytical method involves elution of the organic acids from a column of Dowex 1 with a continuously increasing concentration of formic acid starting at zero and approaching 3.5 N.   Fumaric acid is eluted immediately after citric acid and, if present, forms a clearly defined peak on the titration curve.   Maleic acid requires the use of a considerably higher concentration of formic acid for convenient elution (approaching 6 N) and, even so, is first eluted many fractions after citric acid.

[2] The data for "undetermined acids" represent the sums of the titrations of t fractions which are eluted by formic acid from the Dowex 1 analytical column in a vance of malic acid.   The present "undetermined acids" fraction is usually a mu smaller quantity than has been reported under the same designation in earlier pape and may thus give the misleading impression that the composition of the tobac leaf with respect to organic acids is nearly completely known.   The difference aris from the chromatographic technique now used to determine the so called "tot organic acids."   This method yields data of highly satisfactory precision despite i empirical nature (2).

In explanation, it may be pointed out that such substances as aromatic phenol acids, phosphorylated organic acids, and phosphoric and sulfuric acids (as well oxalic acid) are retained quantitatively by the Dowex column as ordinarily operate Such possible components of the tissue as pyruvic, oxalacetic, glycolic, glyoxyli glyceric, and α-ketoglutaric acids, if still present in the dried leaves, are eluted, b are in part or even entirely destroyed or lost during the evaporation of the form acid in a stream of air at 48° in preparation for titration.   Thus, many of the possib components that were previously wholly or in part included in the titration betwee

15 increased slightly in all of the samples except the water control. This fraction comprises a mixture of about seven components of which only D-glyceric acid and succinic acid have been identified.

The behavior of the protein nitrogen (Line 16) shows the anticipated stimulation of protein metabolism in the leaves cultured in fumarate or succinate as compared with the water control. The unusual observation is that less protein nitrogen disappeared from the leaves cultured in maleate than from the water control. The small quantity of starch present (Line 17) was nearly completely metabolized in all of the samples.

<div align="center">DISCUSSION</div>

Metabolism of Fumaric and Maleic Acids—Data are assembled in Table II from which certain conclusions can be drawn regarding the fate of fumaric and maleic acids in comparison with that of succinic acid when these substances are acted upon by the enzyme systems of tobacco leaves. Line 1 shows the differences (rounded to two figures) between the quantities of the respective acids taken up (Table I, Line 7) and the quantities found in the tissues after 48 hours (Table I, Line 13). These differences represent the amounts of the three acids which disappeared as such and accordingly must have entered into the metabolism. The ratios to the respective uptakes are shown as percentages in Line 2, and it is clear that both fumaric acid and maleic acid are proportionately much less extensively metabolized than succinic acid. It is known from previous work that approximately 90 per cent of the succinic acid supplied at pH 6 under similar conditions is also metabolized (14), and it would thus appear that the extent to which both fumaric acid and succinic acid enter into reaction in the tobacco leaf system is not particularly sensitive to pH in the range between pH 5 and 6. This is not true of maleic acid, and, if the factors used in computing the uptake are reliable, it is of importance to note that *less* maleic acid was taken up at pH 5 than at pH 6, that a smaller *quantity* of this substance was metabolized at pH 5 than at pH 6, and that a smaller *proportion* of the amount taken up was metabolized at pH 5 than at pH 6. Exactly the converse of these three statements has been almost invariably found to be true for malic acid and citric acid, and, within narrow limits, for both fumaric and succinic acids. On these grounds alone, maleic acid is seen to behave in an exceptional manner.

The data for respiration loss (Table II, Line 3) represent the quantities obtained by subtracting the small increases in the organic solids of the tissues, after correction for the carbon dioxide of the ash (Table I, Line

arbitrarily selected pH limits of the material extracted by ether from acidified tissue are no longer included in the present so called "total organic acid" fraction. It is hoped that an adequate definition of this quantity may ultimately be obtained.

5), from the amounts of acid taken up by each sample (Table I, Line 8). Comparison with the respiration loss from the control sample cultured in water, which is merely the corrected loss of organic solids, shows that the general respiration was stimulated about 60 per cent in the leaves cultured

TABLE II

Metabolism of Fumaric, Maleic, and Succinic Acids by Tobacco Leaf System

Data expressed in terms of 1 kilo of initial fresh weight of leaves. For explanations of the derivation of these quantities, see the text.

Line No.		Water	Potassium fumarate		Potassium maleate		Potassium succinate
			pH 5	pH 6	pH 5	pH 6	pH 5
1	Acquired acid metabolized, *m.eq.*		84	81	51	120	209
2	Acquired acid metabolized, as % of uptake		49	52	18	37	92
3	Respiration loss, *gm.*	5.5	8.3	8.4	8.4	9.7	11.6
4	Loss of organic acids, *m.eq.*	8.1	5.9	2.5	32	59	87
5	" " " " *gm.*	0.5	0.34	0.15	1.9	3.4	5.1
6	Acquired acid converted into different acid, *m.eq.*		78	79	19	61	122
7	Acquired acid converted into different acid, *mmoles*		39	39.5	9.5	30.5	61
8	Loss of malic acid, *mmoles*	+31.3	+8.5	+8.6	+9.4	+6.8	−19.2
9	Sum, Lines 7 and 8, *mmoles*		47.5	48.1	18.9	37.3	41.8
10	Increase of citric acid, *mmoles*	16.7	32.3	30.6	7.2	13.7	24.7
11	Ratio of sum to increase of citric acid	1.9	1.5	1.6	2.6	2.7	1.7
12	Sum, Δ malic + Δ citric acids, *m.eq.*	−12.5	+79.8	+74.7	+2.9	+27.4	+112
13	Acquired acid converted into acid other than malic or citric acid, *m.eq.*		−1.6	+4.1	+17	+33	+10
14	Uptake of 0.2 M solution, *ml.*		426	394	724	812	489
15	Change in fresh weight, *gm.*	+167	+78	+54	−489	−469	+129
16	Transpiration, *ml.*		348	340	1210	1280	360

in fumaric or maleic acid and about 100 per cent in those in succinic acid. No distinction between the effects of fumaric and maleic acids can be drawn.

Line 4 of Table II shows the apparent losses of organic acids from the several systems. The data are the differences between the amounts of acid taken up (Table I, Line 7) and the corresponding increases in total organic acid acidity (Table I, Line 9), and are expressed in gm. in Line 5.

It is assumed that no significant fraction of the metabolized organic acid was converted to substances which are unstable or volatile under the conditions of the analytical method. The apparent losses from the leaves cultured in fumaric acid were negligibly small, and, if the sampling and analytical errors are taken into consideration, suggest that, whatever the metabolic fate of the fumaric acid acquired by the leaves may have been, decarboxylation or other reactions which involve loss of base-binding capacity played little if any part in what occurred.

The situation is quite different with both maleic and succinic acids. Substantial losses of titratable acidity took place and, if the highly probable assumption is made that the observed losses actually fell upon these two substances, about 60 per cent of the maleic acid which was metabolized at pH 5 and 50 per cent of that metabolized at pH 6 disappeared completely, as well as 40 per cent of the succinic acid which was metabolized at pH 5. Both of these acids accordingly entered extensively into reactions which involved decarboxylation. There is no evidence regarding the nature of the other products of such reactions although they may well have contributed to the respiratory loss.

Another aspect of the metabolism of these acids is shown in Lines 6 to 11 of Table II. If from the quantities of acquired acid which were metabolized (Line 1) are deducted the quantities of organic acid acidity which disappeared entirely (Line 4), the differences shown in Line 6 represent the quantities of acquired acid which had some other fate. This fate must have been conversion into an equivalent quantity of a different organic acid since there was no change in titration value. It is of interest to pursue the consequences of the assumption that this other acid may have been malic acid, and that malic acid in turn contributed to the formation of citric acid. Malic acid diminished in all of the samples except the one cultured in succinic acid; in this sample it increased, presumably as a result of transformation of some of the acquired succinic acid. Line 7 shows the molar quantities of acquired acid which are assumed to have been converted transiently into malic acid, Line 8 the actual losses of malic acid, and Line 9 the sums of these quantities. Since malic acid increased in the sample cultured in succinic acid, the "loss" of malic acid is treated as a negative quantity. Line 10 shows the corresponding molar increases in citric acid. The molar ratios between the calculated amount of malic acid assumed to have been converted to citric acid and the actual increases in citric acid found are shown in Line 11.

The molar ratio 1.9 obtained from the data of the control leaves cultured in water lends further support to the hypothesis that the over-all reaction which occurs in excised tobacco leaves cultured in water under the present conditions involves the utilization of 2 moles of malic acid for the forma-

tion of 1 of citric acid. A ratio of this magnitude has repeatedly been observed (15). The ratios 1.5 and 1.6 obtained in the experiments with fumaric acid, and 1.7 in that with succinic acid,[3] suggest that, when a large quantity of extraneous acid enters the system, the reactions become so complex that stoichiometric relationships between the initial substrate and the final product are no longer evident. The low ratio indicates, however, that extremely efficient use is made of this substrate.

The ratios of 2.6 and 2.7 obtained from the data for the metabolism of maleic acid indicate that the assumptions that have been made are inadequate to account fully for the behavior of this substance. The constancy of the ratio, in spite of a 3-fold variation in the amount of maleic acid which was presumably metabolized, suggests that there is a real relationship between the metabolism of maleic acid and that of citric acid, but a ratio greater than 2 indicates that a part of the maleic acid had a fate other than conversion through malic acid to citric acid. Data that bear upon this possibility are shown in Lines 12 and 13 of Table II. Line 12 shows the algebraic sum of the changes of malic and citric acid from Table I, Lines 10 and 11. These quantities represent the number of milliequivalents of acidity which were contributed to the formation of citric acid from some source other than malic acid. In Line 13 they are subtracted from the data in Line 6. The differences in the experiments with fumaric acid are negligible, thereby suggesting that all of the fumaric acid which was metabolized to another acid was converted ultimately to citric acid. The difference of 10 m.eq. in the case of succinic acid is also of doubtful significance in view of the analytical and sampling errors involved;[4] but the differences of 17 and 33 m.eq. in the two experiments with maleic acid are a strong indication that a part of the maleic acid which was metabolized underwent conversion to substances other than malic or citric acid. Regardless of the quantitative significance of the data, the evidence seems unequivocal that the metabolism of maleic acid in tobacco leaves is more complex than is that of fumaric or succinic acid.

Maleic Acid As Inhibitor of Enzyme Reactions in Tobacco Leaves—Morgan and Friedmann (16) have shown that maleic acid at 0.08 M concentration produces a marked inhibition of activated papain if the mixture is incubated for a few hours before the protein substrate is added. In order to see whether this result has any bearing on the present observations on tobacco leaves, it first becomes necessary to inquire into the concentration of maleic acid which may have been present in the tissues. At the end of

[3] The value 1.7 confirms the magnitude of the ratio found in four previous experiments with succinic acid (11).

[4] A similar calculation of the data from another experiment in which succinic acid was administered to tobacco leaves (11) gave four values ranging from 5 to 9.5 m.eq.

4S hours, the initial kilo of leaves cultured at pH 5 in maleate weighed 511 gm. and contained 122 gm. of solids; accordingly there were 389 gm. of water present. The leaves then contained 238 m.eq. of maleic acid, and the molar concentration was therefore 0.31 on the assumption that the maleic acid was uniformly distributed in all of this water. The final concentration in the sample cultured at pH 6 was 0.25 M when calculated in the same way. If maleic acid accumulated in these leaves at a continuous rate, and this was observed to be approximately true for the rate of accumulation of fumaric acid, the concentration in the leaves must have been well in excess of 0.1 M for at least 24 hours. This should have provided ample material as well as sufficient time for interaction with any thiol groups present. If, furthermore, the proteolytic enzymes of the tobacco leaf tissue resemble papain with respect to the property of activation by reagents which generate thiol groups and inhibition by reagents which combine with such groups, one would expect to observe some interference with the process of protein hydrolysis which normally takes place in excised tobacco leaves and which is usually easily detectable after culture in water for 24 hours, and invariably so after 48 hours. The data in Line 16 of Table I clearly show that the loss of protein nitrogen was much less than that from the water control when the leaves were cultured in maleate.

Such behavior of the protein is unique in the experience of this laboratory. Tobacco leaves cultured in water for 48 hours normally undergo a loss of protein nitrogen that ranges from 3 to 8 per cent of the amount initially present, and it is known from earlier work (17) that there is a correlative increase in soluble amino nitrogen. An exceptionally vigorous hydrolysis of 13 per cent of the protein has been observed in one instance (18). When cultured in 0.2 M inorganic salts or alkali salts of organic acids, *e.g.* citrate, malate, succinate, fumarate, oxalate, or tartrate, proteolysis is invariably increased and, with a few exceptions, ranges in 48 hours from 10 to nearly 20 per cent. The factor by which the extent of proteolysis is increased over that in the water control may be as great as 5 in a given case and is, with few exceptions, at least 2. Accordingly, a case in which interference is observed with so sensitive an index of general metabolic rate as the extent of hydrolysis of the proteins of the tissues requires explanation. In view of the known properties of maleic acid in other connections, the hypothesis of inhibition of the proteolytic enzymes seems the most likely explanation.

In view of this, the data were examined for other possible examples of inhibitory effects. What appears to be a second instance is provided by the data for the formation of citric acid (Table I, Line 11). In the water control sample, 50 m.eq. of citric acid were formed. Nearly twice as much was found in the samples cultured in fumarate and about 50 per cent more

than in the water control was formed in the sample cultured in succinate. Both of these substrates therefore stimulated the synthesis of citric acid, and the amounts of acquired acid which were apparently used in this process are shown in Table II, Line 6. In the presence of maleate, however, citric acid formation was much diminished at pH 5 and was appreciably diminished at pH 6. Comparison of Table II, Line 6, and Table I, Line 7, shows that only a small fraction of the maleic acid taken up was converted into a different acid whereas about one-half of the fumaric and succinic acids had this fate. The interpretation of these observations as a further example of inhibition of enzyme action by maleate seems reasonable.

Another possible instance is the extent to which malic acid was used up in the course of the formation of citric acid. Table I, Line 10, shows that there was a heavy demand upon the malic acid in the water control sample. The much smaller demand upon malic acid in the samples cultured in

TABLE III

Apparent Inhibitory Action of Maleic Acid on Metabolic Reactions in Tobacco Leaves

	Inhibition by potassium maleate	
	pH 5	pH 6
	per cent	*per cent*
Loss of protein N, *gm*..........	33	75
Formation of citric acid, *m.eq*........	57	18
Utilization of malic " "	67	62

fumarate was interpreted as a possible example of "sparing" action, since practically all of the fumaric acid available for metabolism (Table II, Lines 1 and 6) was apparently used for the sequence of reactions involved in the formation of malic and, subsequently, citric acids. The equally small demand placed upon the malic acid in the samples cultured in maleate may also be interpreted in this way, but, in view of the fact that only a small amount of maleic acid was converted to a different acid and some of this presumably finally to citric acid (Table II, Lines 7 and 9), an interpretation in terms of inhibition seems at least equally likely. The data for the metabolism of succinic acid further emphasize this possibility.

The data which suggest that maleic acid may act as an inhibitor of certain enzymatic reactions in tobacco leaves are summarized in terms of percentage inhibition in Table III. It is implied that the participation of active thiol groups is essential in each of these transformations. The present experiments were not designed to furnish measurements of inhibitory action, and the figures given are accordingly only rough estimates of orders of magnitude. The effects observed did not arise merely from the

severe dehydration which occurred, for a similar set of tobacco leaf samples cultured in oxalate (11) were equally seriously dehydrated during the experiment but showed no such behavior. A specific effect of the presence of maleic acid therefore seems the most reasonable explanation of the observations.

Enzymatic Mechanisms in Tobacco Leaves—As information on the behavior of the organic acids of the tobacco leaf accumulates, the evidence for the presence in this tissue of a number of enzyme systems which produce well known effects becomes more and more impressive. It must be emphasized, however, that proof in any given case calls for nothing less than isolation of the enzyme in substantially purified form and complete reproduction of the reaction *in vitro*. In the efforts to interpret the present results, and in spite of the uncertainties involved, it is assumed that malic acid occupies an intermediate position between citric acid, which seems to be the end-product of certain reactions, and the other components of the several systems. Malic acid is a substance the accumulation or utilization of which is relatively easily demonstrated, since it is a stable substance for which there is an excellent analytical method. That it lies in the chain of products of enzymatic reactions in a position after succinic acid seems probable in view of the accumulation which occurs when succinic acid is furnished to the leaves.

With respect to the relative positions of succinic and fumaric acids, the evidence is less convincing. However, the fact that administration of fumaric acid in the early preliminary experiment led to an accumulation of malic acid suggests that fumaric acid also precedes malic acid in the sequence.

Nevertheless it is not yet possible to conclude that the sequence of reactions is precisely that characteristic of the tricarboxylic acid cycle. Such a conclusion involves acceptance of the view that the sequence is succinic → fumaric → malic → oxalacetic → citric acid. The last step presumably involves a condensation of oxalacetic acid with acetyl coenzyme A. If the interpretation of the observed 2:1 molar relationship between malic and citric acid is valid, the acetyl group of the acetyl coenzyme A must have been provided by a different sequence of reactions into which a substantial fraction of one of the components of the chain had been shunted. As an illustration of the difficulty of this view, the data of the experiment with fumaric acid may be cited. This substance is taken up less extensively than succinic acid; it is far less completely metabolized but it is an even more effective stimulant of the formation of citric acid and, accordingly, must have penetrated rather freely into the cells. The data indicate that 122 m.eq. of succinic acid were converted into another organic acid, 38 m.eq. of malic acid and 74 m.eq. of citric acid being formed. No fumaric

acid was detected. Nevertheless all of this acidity must have passed through the stage of fumaric acid if the above sequence of reactions was followed. In contrast, when 171 m.eq. of fumaric acid were made available, only 84 m.eq. passed through the metabolic process; the remainder accumulated unchanged, but 97 m.eq. of citric acid were formed since some of the malic acid was drawn upon. Fumaric acid supplied from without thus behaved differently from the fumaric acid hypothetically formed during the metabolism of succinic acid. It seems obvious that a general theory of the metabolism of the organic acids of tobacco leaves is still to be sought, although some of the details are becoming clear.

SUMMARY

Fumaric acid and maleic acid were made available in 0.2 M solution at pH 5.0 and 6.0 to tobacco leaves (*Nicotiana tabacum* var. Connecticut shade-grown) by means of the excised leaf culture technique. Succinic acid was similarly administered as a positive control. Fumaric acid was taken up somewhat less freely than succinic acid and about one-half of the quantity acquired by the leaves was metabolized, citric acid being almost the exclusive product. Maleic acid was taken up considerably more freely than succinic acid and the greater part was found unchanged in the tissues. Although a small contribution to the formation of citric acid appears to have been made, much of that part of the maleic acid which was metabolized was converted into substances the identity of which has not yet been established.

Maleic acid present in the tissue apparently behaved as an inhibitor of the activity of the proteolytic enzymes and of the enzyme systems concerned with the formation of citric acid. It also appeared to have interfered with the utilization of malic acid in the transformations that normally occur. The inference may be drawn that the presence of active thiol groups is essential for these activities.

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FORMATION OF PICOLINIC AND QUINOLINIC ACIDS. FOLLOWING ENZYMATIC OXIDATION OF 3-HYDROXYANTHRANILIC ACID*

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Since it is known that 3-hydroxyanthranilic acid (3-HA) is a product of tryptophan degradation (2–5) and that it satisfies the nicotinic acid requirements of several organisms (6–11), it has been generally assumed that the formation of quinolinic acid by oxidation of 3-HA (8, 12, 13) is closely related to nicotinic acid biosynthesis. On the other hand, quinolinic acid has been shown to substitute for nicotinic acid very poorly, if at all (14, 15). Furthermore, it is not known that the biosynthesis of the pyridine derivatives made from the vitamin, nicotinic acid, involves the free acid as an intermediate.

An enzyme system has been described which converts 3-HA to quinolinic acid through an unidentified intermediate with an absorption maximum at 360 mμ (16). In the present work it has been found that in quinolinic acid production only the synthesis of the intermediate is enzyme-catalyzed; the subsequent formation of quinolinic acid is spontaneous. An enzyme which does attack the intermediate has been concentrated from extracts of liver. This enzyme does not make or degrade quinolinic acid, but converts the intermediate to picolinic acid.

Methods and Materials

3-Hydroxyanthranilic acid was obtained commercially and dissolved either in deoxygenated 0.02 M phosphate buffer or in dilute sulfuric acid. The acid solution was used in the later experiments, since it was found that dilute solutions (100 γ per ml.) were stable in 10^{-3} N acid.

The appearance and subsequent disappearance of the intermediate compound were followed in either a Beckman DU spectrophotometer or a Cary recording spectrophotometer at 360 mμ. The disappearance of 3-HA was observed at 317 mμ; this measurement is obscured by the absorption of the intermediate at this wave-length, but is useful in determining the extent of 3-HA utilization when the intermediate is no longer present.

Bioassays for nicotinic acid with *Lactobacillus arabinosus* were kindly carried out by Miss R. C. Gardiner of this Institute.

Picolinic, quinolinic, nicotinic, and isonicotinic acids were obtained commercially. Isocinchomeronic acid was generously provided by Mr. R. W. H. Gentry of E. I. du Pont de Nemours and Company, Inc.

EXPERIMENTAL

3-Hydroxyanthranilic Acid Oxidase—This enzyme has been reported by Priest *et al.* (17) to occur in the livers of various species. This observation has been confirmed, and extracts of acetone powders have been found to contain as much activity as homogenates of fresh liver. It was found unnecessary to observe any temperature control in preparing the acetone powder. Rat livers were blended with 10 volumes of acetone, filtered with suction, blended with acetone a second time, again filtered, and air-dried. Powders prepared in this way or at low temperature gradually lost activity when stored over anhydrous $CaSO_4$ at refrigerator temperature, but retained full activity for months when stored at $-15°$.

Aqueous extracts of the powder (1:10) prepared at room temperature with occasional stirring were used routinely. Although active fractions were obtained with a variety of conventional purification procedures, the yields were not encouraging, and the enzyme became unstable.

The reaction catalyzed by 3-hydroxyanthranilic oxidase is not well defined. The product of the oxidation is an unstable compound that has an absorption maximum at 360 mμ (Fig. 1, *A*). Some properties of the product have been discussed recently by Long *et al.* (18) and by Miyake *et al.* (19). The non-enzymatic nature of the conversion of the compound with 360 mμ absorption to quinolinic acid was discussed previously (20) and was observed independently by Long *et al.* (18). The conversion follows first order kinetics and has a velocity constant of 0.014 min.$^{-1}$ ($t_{\frac{1}{2}} = 43$ minutes) at 25° and pH 9.4 (Fig. 1, *B*).

The rate of the spontaneous conversion of the intermediate to quinolinic acid is not influenced by pH. In acid solutions, however, as noted by others (18, 19), there is a rapid, irreversible conversion to another as yet unidentified compound. With larger amounts of 3-HA, the increased amount of intermediate undergoes a third spontaneous reaction that results in the formation of material with an absorption maximum at 450 mμ.

Because of the limitation imposed by the properties described above, measurement of oxygen consumption could be made only with dilute solutions. Sufficient enzyme to consume all of the 3-HA within a few minutes was used. Various devices were employed to increase the sensitivity of the manometer: the fluid volume was increased to the maximum
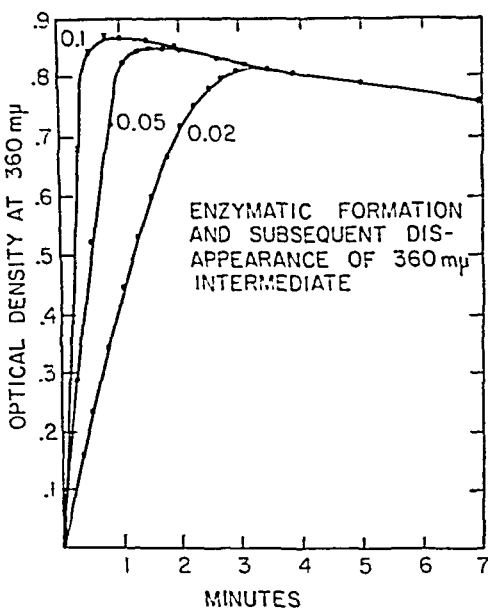
FIG. 1, A
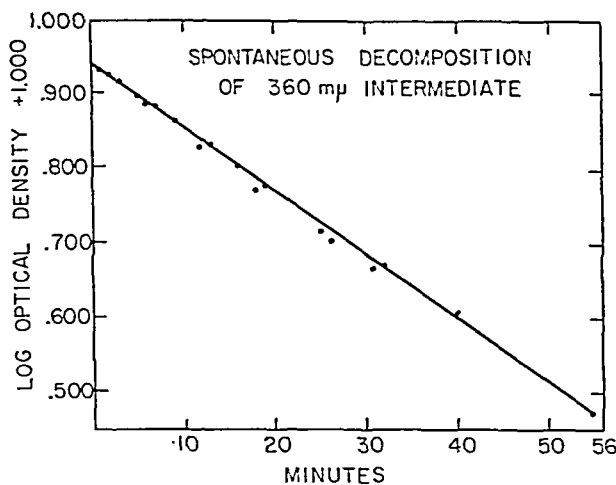
FIG. 1, B

FIG. 1. A, curves showing the rate of appearance of the 360 mμ intermediate with the ml. of enzyme indicated. The reactions were carried out in 3 ml. of total volume with 50 μmoles of phosphate, pH 7.4, and 10 γ of 3-HA in cells with 1 cm. light path. In each case blanks without 3-HA were used as reference cells. In B, the concentration of intermediate is plotted as the log of optical density, and the portions of all three curves following maximal optical density are included.

permitted by vessel design; decane was used as the manometer fluid. A series of determinations employing the bubble method of optical lever (21) was made in collaboration with Dr. D. Burk. While time lags of the order of minutes prevent precise correlation of oxygen consumption with intermediate formation, it is apparent that under these conditions 2 atoms of oxygen are consumed rapidly and only a small part of the intermediate

TABLE I

Oxygen Consumption during Formation of Intermediate

To each vessel were added 100 μmoles of pyrophosphate, pH 9.4, 0.5 ml. of 3-hydroxyanthranilic oxidase (side arm), water to give a final volume of 8 ml., and 3-HA, 0.1 or 0.2 ml. of a solution containing 1 mg. per ml. The gas phase was air; temperature, 25°. The values are expressed in micromoles per vessel.

3-HA added	0.65	1.3
O$_2$ consumed*	0.7	1.7
Intermediate produced†	0.6	1.2

* Oxygen consumption was completed within 6 minutes after tipping in the enzyme. The values are corrected for changes in a blank without substrate.

† Calculated from optical densities at 360 mμ. Aliquots were diluted 6-fold and read against a similar dilution from the vessel without substrate. The values were obtained 10½ minutes after tipping and are corrected for spontaneous decay.

TABLE II

Inhibition of 3-Hydroxyanthranilic Oxidase by Cyanide Ion

In each cuvette were placed 0.5 ml. of 0.1 M buffer, either phosphate, pH 7.4, or pyrophosphate, pH 9.4, 0.05 ml. of enzyme, water to give a final volume of 3.0 ml., sodium cyanide to give the concentration indicated, and, at zero time, 0.1 ml. of a solution containing 100 γ of 3-HA per ml. The increments in optical density for the time between ½ and 1½ minutes are indicated.

Total cyanide concentration	10^{-2} M	3.3×10^{-3} M	10^{-3} M	3.3×10^{-4} M	10^{-4} M	0
pH 7.4 . .	0.217	0.280	0.370		0.370	0.370
" 9.4 . ..	0.009	0.038	0.095	0.150	0.210	0.210

formed has disappeared during this time (Table I). The addition of large amounts of catalase, or of catalase plus ethanol, did not influence the rate or extent of oxygen consumption. This is evidence that hydrogen peroxide is not involved in the oxidation of 3-HA (22). Therefore the suggestion of Long *et al.* (18) is supported, that 2 atoms of oxygen are consumed in the formation of the intermediate, which is thus at the oxidation level of quinolinic acid.

This conclusion is further supported by the observation that the rate of disappearance of the 360 mμ absorption is the same in the presence or absence of oxygen. These observations were made with reaction mixtures

incubated in modified Thunberg tubes which were read directly in the Beckman spectrophotometer in a carriage built to hold three such tubes.

It has been variously reported that the oxidation of 3-HA is and is not sensitive to cyanide (20). The present studies indicate that the cyanide ion is a competitive inhibitor of 3-HA (Tables II and III). Therefore, at pH values near neutrality very high concentrations of cyanide are required to demonstrate any inhibition, whereas at values near pH 9 the inhibition is 100 times as great.

3-Hydroxyanthranilic oxidase is inhibited by p-chloromercuribenzoate, which is evidence for the requirement of SH groups. This is supported by the sensitivity of the enzyme to hydrogen peroxide (1).

TABLE III

Competitive Nature of Cyanide Inhibition

In each cuvette were placed in order 0.5 ml. of 0.1 M pyrophosphate buffer, pH 9.4, 0.05 ml. of enzyme, water to make the final volume 3.0 ml., cyanide, and at zero time, 3-HA. Optical densities were recorded in a Beckman DU spectrophotometer at 360 mμ in 1 cm. cuvettes. The rates recorded are for the period between 30 seconds and 1 minute after adding the substrate.

CN⁻ \ 3-HA	0	2×10^{-3} M	2×10^{-4} M	2×10^{-5} M
M				
2×10^{-5}	0.120	0.020	0.080	0.120
2×10^{-4}	0.200	0.070		
4×10^{-4}	0.200	0.090		

Enzymatic Utilization of Intermediate—The rate of disappearance of the 360 mμ absorption is completely uninfluenced by the amount of extract of acetone powder used for its formation. Additions of more extract following its formation likewise do not influence the rate of removal of the 360 mμ absorption. Incubation mixtures deproteinized with zinc acetate (excess zinc removed as $Zn(OH)_2$) show the same rate of decrease of the 360 mμ band, and, again, the rate is not influenced by the addition of extracts of acetone powders. On the other hand, extracts of fresh or frozen liver, either rat, guinea pig, or beef, accelerated the rate of disappearance of the 360 mμ absorption. The most consistent activity was found in guinea pig liver, although an active preparation has been purified from beef liver. As will be described, the product of this enzyme acting on the intermediate is picolinic acid. Since the substrate and type of reaction carried out by this enzyme are not known, at this time no descriptive name will be applied. In the remainder of this article, the enzyme will be referred to as Enzyme II.

Purification of Enzyme II—The 360 mμ-removing activity is weak in the best livers assayed. The following procedure was found convenient for the concentration of Enzyme II. Guinea pig livers were blended with 4 volumes of cold 1.25 per cent KCl, and the homogenate was filtered through cheese-cloth. All subsequent operations were carried out near 0°. The homogenate was brought to pH 4.9 to 5.0 by the addition of 1 N acetic acid with mechanical stirring. The mixture was centrifuged, and 120 to 125 gm. of ammonium sulfate were added per liter of supernatant fluid. After stirring the mixture mechanically for 15 minutes or more, the precipitate was removed by centrifugation in a large refrigerated angle head (4000 × g). Another 120 gm. of ammonium sulfate were added per liter of original acid supernatant fluid, and the precipitate was centrifuged as before. The second ammonium sulfate precipitate was dissolved in water with a small amount of phosphate, pH 7.4. This solution was re-

TABLE IV

Purification of Enzyme II

	Ml	Units per ml	Mg. per ml	Specific activity*	Total units
Original extract	1,200	25	44 5	0.56	30,000
pH 5 supernatant	895	25	18	1.4	22,375
1st (NH₄)₂SO₄ fractionation	28.5	400	93	4.3	11,400
Cγ eluates	40	250	9.2	27	10,000
2nd (NH₄)₂SO₄ fractionation	10	410	10.8	38	4,100

* Specific activity is defined as units per mg. of protein.

fractionated with ammonium sulfate between approximately the same concentrations as before, correcting roughly for the ammonium sulfate retained by the precipitate. The second precipitate was dissolved in water and dialyzed with rocking against running distilled water for 3 to 4 hours. Prolonged dialysis caused significant inactivation. 65 ml. of alumina Cγ, 11 mg. of solids per ml., were added per 100 ml. of dialyzed enzyme. After a few minutes stirring, the mixture was centrifuged, and an additional 40 ml. of Cγ were added. Each Cγ precipitate was washed by suspension in 5 volumes of cold distilled water and centrifugation. After two washes, the enzyme was eluted with two portions of 0.5 M potassium phosphate, pH 7.8, with a total volume about equal to that before addition of alumina. The pooled Cγ eluates were fractionated with ammonium sulfate, and the fraction obtained between the addition of 33 and 52 gm. per 100 ml. of eluate was collected. This was dialyzed briefly as before. This solution, as well as all preceding ones, retained full ac-

tivity when frozen at −15°, but lost activity gradually at refrigerator temperatures. The purification is summarized in Table IV.

Properties of Enzyme II—Enzyme II acts on the unstable product formed by 3-hydroxyanthranilic oxidase. It is possible to deproteinize incubation mixtures of the oxidase and use the resulting solution as substrate for Enzyme II, but, since the extracts of acetone powders used had no effect on the disappearance of the intermediate, the second enzyme was routinely assayed in the presence of the first. The incubation mixture
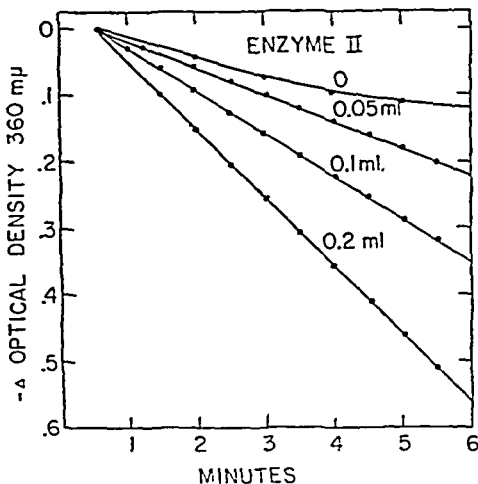

Fig. 2. Enzymatic utilization of 360 mμ intermediate. The 360 mμ intermediate was made by adding 0.1 ml. of a solution containing 100 γ of 3-HA per ml. to a cuvette containing 0.1 ml. of 3-hydroxyanthranilic oxidase, 50 μmoles of pyrophosphate, pH 9.4, and water to give 3.0 ml. when Enzyme II was added. Enzyme II in the quantitites indicated was added 1 minute after 3-HA, and the optical density was read at ½ minute intervals thereafter *versus* a cuvette containing all additions except 3-HA.

contained 0.5 ml. of 0.1 M pyrophosphate buffer, pH 9.4, 0.1 ml. of the oxidase, Enzyme II, and water to 2.9 ml. At zero time 0.1 ml. of 3-HA, 100 γ per ml., was added. The reaction was followed at 360 mμ either in cuvettes with 1.0 cm. light path or in Pyrex test-tubes with an apparent path of 1.3 cm. The formation of the intermediate was maximal in about 1 minute, and the initial rate of decline in optical density determined in the succeeding several minutes was used as a measure of Enzyme II. This rate is not proportional to enzyme concentration until corrected for the spontaneous decomposition of the intermediate (see Fig. 2). A unit of enzyme was arbitrarily defined as the amount causing an initial decline in density of 0.001 per minute in excess of the spontaneous rate when measured with a 1 cm. light path.

The reaction catalyzed by Enzyme II does not depend on the presence of oxygen or any additional substance. In contrast to 3-hydroxyanthranilic oxidase, Enzyme II is inhibited approximately 50 per cent by 10^{-5} M HCN. Since there is little effect of pH on the degree of inhibition between pH 9.4 and pH 7.4, it appears that HCN, not CN^-, is the inhibitor. p-Chloromercuribenzoate also inhibits Enzyme II. Enzyme II exhibits a broad range of maximal activity between pH 6 and 9.5, and the activity declines sharply at more acid and alkaline pH values. Even with the low concentrations of substrate used, Enzyme II appeared to exhibit zero order kinetics until about half of the intermediate was removed. Since the initial concentration of 3-HA was only about 2×10^{-5} M, it is apparent that Enzyme II has a strong affinity for the intermediate.

Nature of Product of Enzyme II—A possible relationship between the product of Enzyme II and nicotinic acid was explored by assaying the incubation mixture and a corresponding blank (without 3-HA) for niacin activity with *L. arabinosus*. No increase over the blank value was found on direct analysis or after autoclaving in 66 per cent acetic acid.

In order to determine the nature of the product of Enzyme II, its ultraviolet spectrum was measured. This was done without prior deproteinization, to avoid the possibility of altering the product through chemical manipulation. The intense absorption of ultraviolet light by protein and nucleotides makes it impossible to determine the spectrum of the product of Enzyme II activity with a single monochromator spectrophotometer. However, the double monochromator of the Cary spectrophotometer permits measurements to be made in the presence of the amounts of enzyme used (23). The spectrum so obtained shows a maximum at 264 mμ. After deproteinizing with zinc, trichloroacetic acid, or perchloric acid, the spectrum (taken at neutral pH) exhibits the same 264 mμ maximum.

To obtain enough of the product for identification, a large scale preparation was made. The incubation mixture consisted of 200 ml. of dialyzed second ammonium sulfate precipitate of Enzyme II (90,000 units), 5 mmoles of pyrophosphate buffer, pH 9.4, 1 ml. of 3-hydroxyanthranilic oxidase, and water to give 600 ml. 3-HA was added in solution, approximately 3.5 mg. at a time. The reaction was followed by reading aliquots at 360 mμ and at 317 mμ. Under these conditions only small increases in the 360 mμ reading ($\Delta < 0.4$) were found, whereas the 317 mμ readings decreased steadily. When the 360 mμ reading decreased to values near zero (approximately 30 minutes), more substrate was added. It was found necessary to add fresh oxidase with each addition of substrate, since the oxidase became inactivated during the reaction. Eventually about 35 mg. of 3-HA were added. 30 gm. of trichloroacetic acid were added, and the mixture was left in the refrigerator overnight. The

precipitate was removed by filtration, and the filtrate was extracted four times with amounts of ether somewhat less than equal to the volume of the filtrate. The aqueous phase was concentrated by distillation *in vacuo.*
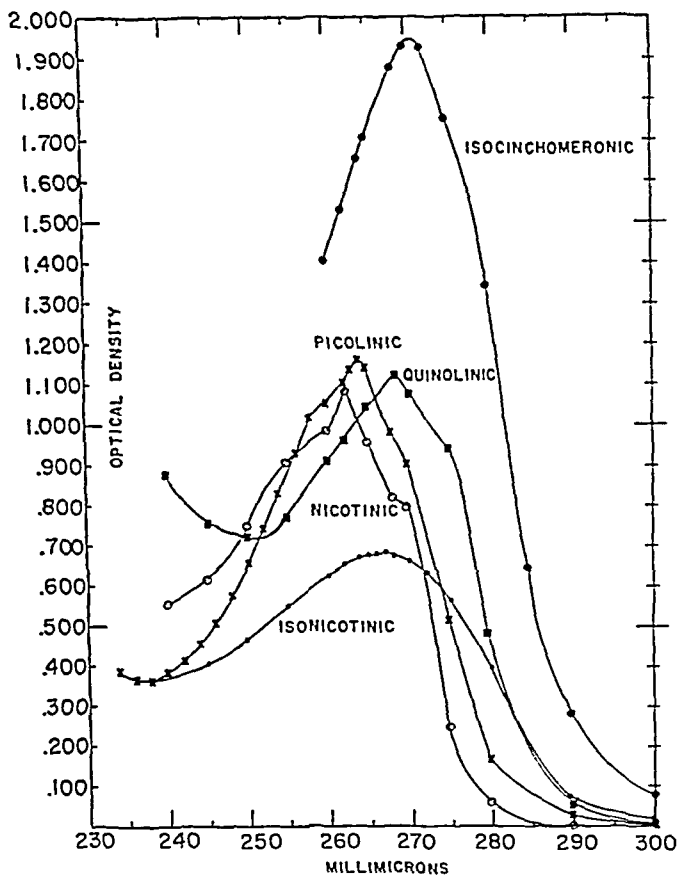
Fig. 3. Absorption spectra of pyridinecarboxylic acids. Each compound was present at 3.3×10^{-4} M concentration in phosphate buffer, pH 6.8.

The product was purified by chromatography with ion exchange resins. Originally the 264 mµ peak was not absorbed by either anion or cation exchangers. By exchanging other cations for H⁺ with Dowex 50 H and then replacing most of the anions in the mixture by acetate with Dowex 1 acetate, relatively pure solutions of the product in acetic acid were obtained. The acetic acid was removed by vacuum distillation, and the residue was chromatographed on Dowex 50. HCl eluates from Dowex 50 were concentrated *in vacuo* and dried, leaving a crystalline residue that

appeared to be a salt. The product was extracted from the salt with absolute ethanol.

The yield of product, based on the optical density at 264 mμ of dilutions of the ethanol solution in buffer, was 18 mg. or 64 per cent of the 3-HA added. On concentrating the ethanol, the product deposited on the vessel as a gum, but a white solid was obtained by precipitation from ethanol with ether. This solid was used for the infra-red analysis.
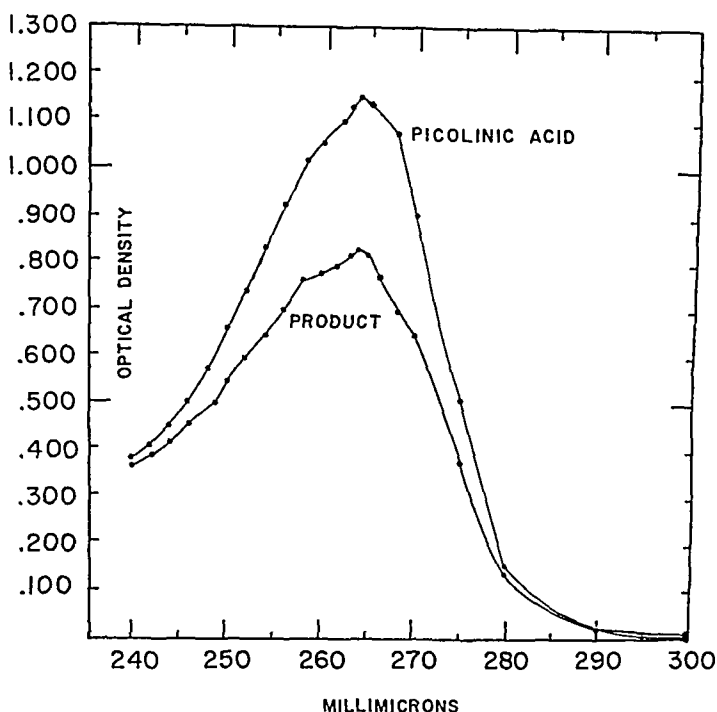
Fig. 4. Absorption spectra of enzyme product and picolinic acid. The final ethanol extract was diluted 60 times in dilute phosphate buffer. Both compounds were at pH 6.8.

Since the amount of material obtained was not sufficient for a conventional melting point determination, a Kofler stage was used. Whereas authentic picolinic acid hydrochloride softens below 200° and melts with decomposition around 225° when heated in a capillary, on the Kofler stage it decomposes without leaving a residue at 177–182°. The isolated material darkened around 180°, but left a residue which did not melt.

The similarity of the spectrum of the product to those of nicotinic and quinolinic acids encouraged comparison with other pyridine derivatives. It is apparent (Figs. 3 and 4) that the spectrum coincides with that of picolinic acid and is clearly distinguished from all others. On acidification both the product and picolinic acid continue to exhibit maximal absorption at 264, but the extinction increases 1.7 times in each case. As shown in

Table V, chromatography on paper separated the three pyridine mono-carboxylic acids, but failed to separate the enzyme product from picolinic acid. Infra-red spectra confirmed the identification.[1]

If the pyridine ring were formed prior to decarboxylation, it might be supposed that either quinolinic acid (pyridine-2,3-dicarboxylic acid) or isocinchomeronic acid (pyridine-2,5-dicarboxylic acid) would be an intermediate. Since the spectra of these compounds do not appear during incubation, if they occur they must be metabolized rapidly. When these compounds were incubated with Enzyme II, there was no detectable change in their spectra.

TABLE V

R_F Values of Pyridine Monocarboxylic Acids

	tert-Butanol 70, formic acid 15, H₂O 15	sec-Butanol 75, formic acid 15, H₂O 10	Acetone 80, H₂O 20
Picolinic acid.....................	0.61	0.58	0.50
Nicotinic "	0.81	0.83	0.90
Isonicotinic acid	0.68	0.72	0.33
Product..........................	0.62	0.58	0.50

Chromatography was carried out with an ascending method on Whatman No. 3 paper. Spots were visualized as dark areas when the paper was irradiated with a Mineralight ultraviolet source. The solvents used have been described previously (24–26).

DISCUSSION

The nature of the oxidation of 3-HA has been considered by Crandall (27) to be similar to the oxidations catalyzed by certain other Fe⁺⁺-containing oxidases; namely, those oxidizing homogentisic acid, protocatechuic acid, and catechol. Experiments reported above are in agreement with this suggestion in so far as 2 atoms of oxygen are consumed and no H_2O_2 appears to be formed.

The structure of the intermediate can only be surmised. It has been reported by several groups of investigators that the intermediate is irreversibly altered by acid pH. Miyake et al. (19) have prepared a derivative of the acid decomposition product, and Long et al. (18) have also observed reactions with carbonyl reagents following acidification. Wiss (28) claims to have prepared a dinitrophenylhydrazone of the true intermediate, but his note does not establish that his derivative is formed from the true intermediate rather than from the acid decomposition product.

[1] I am indebted to Mr. W. M. Jones of this Institute for recording the infra-red spectra.

The structures proposed by Miyake *et al.* for the intermediate and the acid decomposition product are inconsistent with the indications that 2 atoms of oxygen are consumed in the oxidation of 3-HA and none in the conversion of the intermediate to quinolinic or picolinic acid.
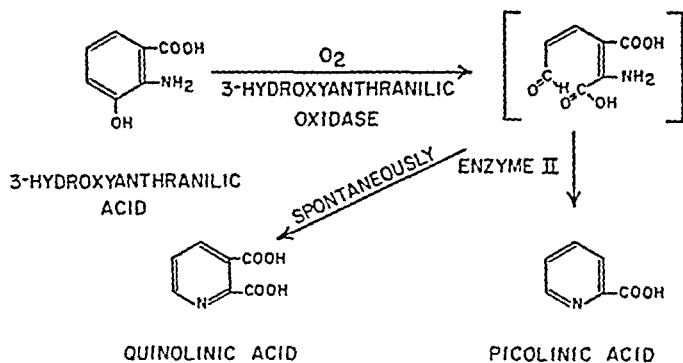
The identification of picolinic acid as the product of Enzyme II raises two questions: (1) Is this a normal metabolic product, and if so, does it have a function, and (2) what implications does this have for nicotinic acid biosynthesis? Free picolinic acid has not been identified as a component of natural systems, but *N*-methylpicolinic acid, called homarine, has been found in several marine organisms in large quantities (29, 30). The function, origin, and fate of homarine are unknown. The low level of Enzyme II in liver cannot be considered evidence against its physiological function, since this entire pathway, starting with tryptophan, acts very slowly compared with many other systems, and at least one prior step, the introduction of the hydroxyl group, has never been obtained clearly in cell-free systems. Since 3-HA may be formed very slowly under normal circumstances, the amounts of Enzyme II that occur in liver may be sufficient to handle a large portion of its oxidation product.

The biosynthesis of nicotinic acid remains very obscure. It is still tempting to retain a unitary mechanism for obtaining pyridine rings, but it must also be considered that the oxidation to the 360 mμ-absorbing compound initiates a pathway which has no relation to nicotinic acid synthesis. In experiments in which 3-HA (or a precursor) is added to liver preparations or is administered to whole animals, the oxidase is able to react much faster than with the amount of substrate usually available, and therefore forms large amounts of 360 mμ intermediate. Since Enzyme II is much slower in its activity, the bulk of the intermediate goes spontaneously to quinolinic acid, which is an inert end-product. It is, of course, conceivable that still another enzyme exists which converts the intermediate to nicotinic acid.

Alternative mechanisms would involve other hypothetical reactions of 3-HA. One possibility is amidation prior to oxidation. Although it is known that nicotinic acid is equivalent to nicotinamide in vitamin activity, it is possible that in the biosynthetic pathway only the amide, and not the free acid, is formed. Similarly, a riboside or ribotide may be formed before oxidation. These two types of substitution of 3-HA are not exclusive. Both represent reactions which must occur at some stage in the metabolism of nicotinic acid, since the free acid has no known biological function, but is found as a component of the pyridine nucleotides.

Without knowledge of the structure of the intermediate, the nature of the reaction catalyzed by Enzyme II cannot be ascertained. The most reasonable substrates are the often suggested orthoquinone of 3,4-dihydroxyan-

thranilic acid and the corresponding hydrolytic product, the open chain dicarboxylic acid amino aldehyde. Since quinolinic acid, and not isocinchomeronic acid, can be formed, it seems reasonable to assume that only the 3,4 split in the 3-HA ring occurs. If the open chain compound is indeed the intermediate, it is probable that the rate-limiting reaction in its spontaneous disappearance is an isomerization of the double bond to place the carboxyl groups in the cis configuration. This would be followed by a rapid condensation to form the stable pyridine ring. The reaction of Enzyme II almost necessarily involves decarboxylation. It is possible that in the process of decarboxylation the rotation of the bond to put the amino group in a position to condense to form the pyridine ring might be favored or compelled. Thus ring formation and decarboxylation might appear as simul-

taneous reactions catalyzed by one enzyme. These reactions are indicated in the accompanying diagram.

SUMMARY

The metabolism of 3-hydroxyanthranilic acid by liver preparations takes place in two steps. In the first, the substrate is oxidized with the utilization of 2 atoms of oxygen to form a compound that absorbs maximally at 360 mμ. This reaction is competitively inhibited by cyanide ions. The second step may be in at least two directions. A non-enzymatic reaction may occur to form quinolinic acid. An enzyme has been described which attacks the intermediate to form picolinic acid. The nature of these reactions and their relation to nicotinic acid synthesis are discussed.

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POLYUNSATURATED FATTY ACIDS IN NORMAL HUMAN BLOOD

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Information on the fatty acid composition of human blood has been fragmentary and conflicting (1, 2). Wilson and Hansen (3) have reported unsaturated fatty acids in human plasma and suggested an average iodine number of 108. In 1937, Brown and Hansen (4) showed differences in the amounts of linoleic and arachidonic acids in sera of normal and eczematous children, and later Brown and others (5) demonstrated a decrease of plasma unsaturated acids in the adult man during a prolonged low fat diet. The lipide of human blood cells has been less extensively studied. Analyses by Erickson and coworkers (6) have shown that practically all blood cell lipide is in the stroma, mainly as phospholipide. Wiese and Hansen in 1953 (7) reported a semimicromethod for estimation of blood serum unsaturated fatty acids, but at that time did not possess constants for the pure natural acids and did not clearly characterize human blood lipides. The present availability of an adequate spectrophotometric method for the quantitative determination of polyunsaturated fatty acids suggested that a reinvestigation of human blood fats might be fruitful.

Methods

All subjects were young male medical students in the postabsorptive state (10 hours without food). From each of five subjects, 100 ml. of blood were drawn via the antecubital vein into heparinized syringes and immediately centrifuged. From two other donors a total of 910 ml. of whole blood was transferred by sterile technique to a sterile bottle to which had been added 240 ml. of a solution containing 3.17 gm. of sodium citrate, 1.15 gm. of citric acid, and 3.62 gm. of dextrose. After being mixed, 500 ml., containing 396 ml. of whole blood, were withdrawn immediately for extraction and analysis (Table I, Analysis 1). The remaining 514 ml. of blood were extracted and analyzed separately (Table

* One of the branches of Utilization Research, Agricultural Research Service, United States Department of Agriculture.

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I, Analysis 2). Neither the anticoagulant nor the amount of fatty acid extracted appeared to affect the analyses, and in our final evaluation all data were given the same weight. Plasma and blood cell fatty acids were extracted from each of the six blood specimens and were each analyzed in duplicate. In every case, plasma and cells were separated by centrifuging the whole blood specimens at 2000 r.p.m. for 1 hour. The plasma was pipetted off, and the cells were washed by resuspending and centrifuging them twice in mammalian Ringer's solution. Both plasma and blood cells were subjected to alcohol-ether extraction. Each was initially mixed with 95 per cent ethanol and heated to 60° under a partial vacuum to near dryness. The same procedure was repeated twice with ethanol-ether (3:1). The residue was transferred to a mortar, thoroughly ground with anhydrous sodium sulfate and ether, and filtered through a Büchner fun-

TABLE I

Comparative Analyses of Plasma Fatty Acids on Pooled Blood from Two Persons

The data, except iodine numbers, are expressed as percentages of total fatty acids.

Analysis No.	Linoleic acid	Linolenic acid	Arachidonic acid	Pentaenoic acid	Hexaenoic acid	Oleic acid	Total unsaturated acid	Total saturated acid	Iodine No.
1	22.72	1.85	5.43	0.91	1.21	36.2	68.3	31.7	106.2
2	23.85	2.57	5.73	0.91	1.28	33.2	67.5	32.5	108.8

nel. The processes of grinding and filtration were repeated six to eight times. The filtrate was evaporated first over a steam bath and then under reduced pressure to constant weight. After saponification, the unsaponifiable material was removed, and the fatty acids were extracted with petroleum ether by the method of Kerr and Sorber (8), as modified by Jamieson (9). After evaporation of the petroleum ether, the fatty acids were dried under a vacuum to constant weight and analyzed spectrophotometrically by the method of Herb and Riemenschneider (10). Iodine numbers were determined according to Wijs (11). At all times, except during the extraction and evaporation of solvent, precautions were taken to protect the samples from oxidation by keeping them under special oxygen-free nitrogen and storing them at 4°.

Calculations for the percentages of the individual polyunsaturated fatty acids were made according to Herb and Riemenschneider (10), as modified by Hammond and Lundberg (12) for the inclusion of hexaenoic acid. In the formulas used in the present work, the assumption was made that the pentaenoic acids consist of 50 per cent docosapentaenoic and 50

per cent eicosapentaenoic acids.   The average differences between duplicate analyses of the fatty acids extracted from plasma and blood cells, in per cent, were as follows: linoleic 0.45, linolenic 0.18, arachidonic 0.31, pentaenoic 0.12, and hexaenoic 0.28.   On the basis of these data and those of Table I, we believe our analyses for linoleic acid in plasma are accurate to

TABLE II

Fatty Acid Composition of Human Blood Plasma and Cells

The values for individual fatty acids are expressed as percentages of total fatty acids.

Subject	Whole blood	Total fatty acids	Iodine No.	Linoleic acid	Linolenic acid	Arachidonic acid	Pentaenoic acid	Hexaenoic acid
	Plasma							
	ml.	*mg.*		*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
P. Y.	96	109	105.2	25.3	0.54	6.54	1.67	3.14
W. B.	104	106.7	114.5	26.2	0.85	7.82	1.31	2.08
R. M.	102	117.0	110.0	28.3	0.81	7.77	1.28	2.42
G. K.	122	98.4	108.8	25.9	1.21	7.47	1.75	2.46
P. C.	112	83.4	105.3	21.7	1.60	7.78	1.78	2.61
Average*...... ..			108.3	25.0	1.14	7.14	1.45	2.32
	Cells							
P. Y.	96	23.1		6.62	−0.11	9.34	2.68	5.59
W. B.	104	97.3	95.6	6.09	−0.43	13.1	2.53	3.47
R. M.	102	62.8	97.7	8.09	−0.58	13.5	2.89	4.62
G. K.	122	85.6	98.7	9.29	0.52	11.8	2.73	3.13
P. C.	112	57.5	96.3	8.27	−0.17	14.3	3.16	4.22
W. Z. R. S.	396	285.4	115.4	8.68	−0.02	16.8	3.20	3.63
Average...			100.7	7.84	0.00	13.1	2.87	4.11

* Includes data from Analysis 1, Table I.

within ±1 per cent, and are within a fraction of 1 per cent for any of the other plasma polyunsaturated fatty acids.   Accuracy for blood cell fatty acids is probably of the same order.

RESULTS AND DISCUSSION

The percentage distributions of the individual fatty acids from blood cells and plasma are characteristically different, despite the fact that their iodine numbers are of the same order of magnitude (Table II).   Of the poly-

unsaturated fatty acids, linoleic is predominant in plasma and arachidonic in blood cells. The small negative values for linolenic acid obtained in the analyses of blood cell fatty acids are probably within the limits of experimental error and indicate that essentially no linolenic acid is present. Pentaenoic and hexaenoic acids appear in both plasma and cells in more than trace quantities; to our knowledge there have been no quantitative analyses of these acids reported for human blood. The data for oleic acid are less precise than for the polyethenoid acids because of the difficulty in making accurate iodine number determinations on small fatty acid samples. For the same reason the values for total unsaturated and total saturated acids are less exact than those for the individual polyethenoid acids. Of the total plasma fatty acids, oleic acid averaged 21.1, total unsaturated acids 58.1, and total saturated acids 41.9 per cent. Of the total blood cell fatty acids, oleic averaged 11.3, total unsaturated 40.1, and total saturated 59.9 per cent.

Although the data obtained in these analyses are not extensive, we believe that they are of interest because they permit a more definite statement of the amounts and variability of individual blood polyunsaturated fatty acids than has hitherto been made. With blood levels approximately established, some new problems arise and numerous old ones present themselves for reexamination. The large amount of arachidonic acid in blood cells suggests that the formed elements of the blood may be a site for synthesis of this polyethenoid acid. In addition, the oxygen consumption of human serum *in vitro* in the presence of weak ammonium hydroxide and potassium ferricyanide, suspected by Litarczek (13) as being due to autoxidation of unsaturated fatty acids, might profitably be studied again.

SUMMARY

1. Spectrophotometric analyses have been made to determine the amounts of polyunsaturated fatty acids in the blood of seven normal human males in the postabsorptive state.

2. The data suggest that there is a characteristic distribution of polyethenoid acids in the fatty acids of both plasma and blood cells. Linoleic acid is the predominant polyunsaturated fatty acid in the plasma, whereas arachidonic acid is predominant in the cells.

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THE INCORPORATION OF LABELED GLYCINE INTO ERYTHROCYTE GLUTATHIONE

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In a previous report from this laboratory it was shown that the glutathione (GSH) content of rat erythrocytes follows closely the supplementation and withdrawal of dietary methionine (1). This observation suggested that GSH can be synthesized in mature mammalian cells, although such cells contain no nuclei and might, therefore, be expected to synthesize the tripeptide mainly, if not exclusively, during some earlier stage in development (2). Further evidence for GSH formation is found in the experiments of Anderson and Mosher (3), who detected S^{35} in the erythrocyte GSH of rats following the administration of labeled DL-cystine by stomach intubation.

The present work was undertaken to study the rate of incorporation of labeled glycine into the GSH of intact rat erythrocytes. An experiment was also carried out with avian blood in order to compare the activity of non-nucleated with that of nucleated cells. In both cases rapid incorporation was observed.

EXPERIMENTAL

The radioactive amino acids used were glycine-1-C^{14} (Tracerlab) and uniformly labeled L-glutamic acid (Nuclear Instrument and Chemical Corporation).

All experiments were carried out in vitro. Erythrocytes from freshly drawn heparinized blood were incubated with the labeled amino acids in a Warburg apparatus. The blood samples were obtained by heart puncture from male Sprague-Dawley rats weighing between 200 and 300 gm. and by venipuncture from medium sized ducks. After centrifuging the blood and removing the plasma and buffy coat, the cells were washed once with 2 volumes of Krebs-Ringer-phosphate solution (4), to which had been added 80 mg. per cent of glucose (subsequently referred to as Krebs-Ringer solution). A measured volume of the washed cells was then mixed with a double volume of the Krebs-Ringer solution and the cell suspension was transferred to a flask in a 37° Warburg bath. The labeled amino acid, dissolved in a predetermined volume of Krebs-Ringer solution, was added

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to the cell suspension in the flask and the whole was shaken throughout the incubation period at a frequency of 75 cycles per minute.   In certain experiments non-labeled amino acids were also added to the reaction mixture, and in one experiment various concentrations of plasma served as the suspension medium.   All reaction mixtures had a hematocrit value of 0.20.   Unless otherwise stated, an anaerobic system was employed, the gas phase consisting of 5 per cent carbon dioxide and 95 per cent nitrogen.

At the end of the incubation period, 2 ml. aliquots of the reaction mixture were withdrawn and deproteinized with 10 per cent trichloroacetic acid.   The precipitated protein was removed by centrifugation and 25 mg. of GSH were added to each 2 ml. sample to act as carrier.   GSH was then precipitated as the cadmium salt and purified by reprecipitation as the cuprous mercaptide, as described by Waelsch and Rittenberg (5). Suspensions of the mercaptide in absolute ethanol were deposited for counting on aluminum disks by means of the plating procedure of Hutchens, Claycomb, Cathey, and Van Bruggen (6).

Radioactivity determinations were made in a gas flow counter.   The counting times were sufficient to give a standard error of 2 per cent or less.   Corrections for self-absorption were determined from the loss of radioactivity of GSH cuprous mercaptide layers of graded thickness.   The amount of GSH formed was calculated from the equation of Bloch (7).

In order to determine the location of the label in the GSH molecule, samples of the mercaptide were treated with hydrogen sulfide to remove the copper and were then hydrolyzed by boiling in dilute HCl.   The resulting products were separated as dinitrophenylamino acids by adsorption chromatography on silicic acid columns (8).   In a typical experiment, no radioactivity was detectable in the cysteine or in the glutamic acid, but the glycine fraction gave over 800 c.p.m.

GSH concentrations were determined by the nitroprusside method of Grunert and Phillips (9) and total non-protein sulfhydryl groups by the amperometric technique of Benesch and Benesch (10).

All concentrations of amino acids given in the present paper represent the amounts of added amino acids only.   Since free amino acids are present in blood cells, the reported concentrations must be less than the actual values, and, accordingly, the amounts of incorporation into GSH as calculated are correspondingly low.   The error should be small, however, except in the lowest concentrations employed.

Results

Effect of Glycine Concentration—The incorporation of labeled glycine into erythrocyte GSH under varying concentrations of glycine in the presence and absence of added cysteine and glutamic acid is demonstrated

in Fig. 1. It will be noted that the rate of GSH formation increased with increasing glycine concentration and that the simultaneous addition of cysteine and glutamic acid greatly stimulated the formation of the tripeptide. In other experiments it was found that the separate addition of cysteine and glutamic acid had much less effect, cysteine increasing somewhat the formation of GSH and glutamic acid having little if any action within the range of concentrations studied.
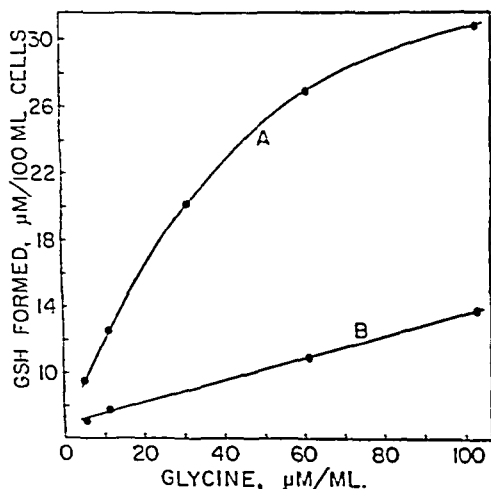

FIG. 1
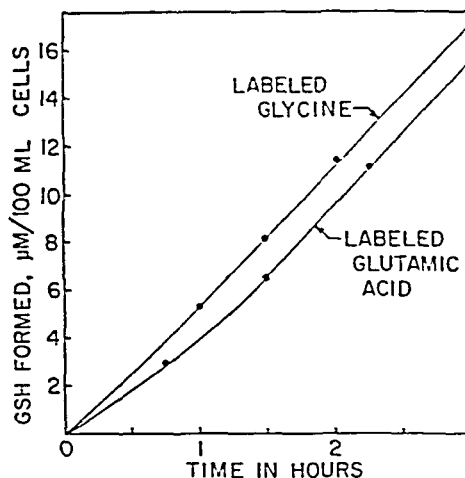
FIG. 2

FIG. 1. Effect of varying concentrations of glycine on glutathione formation in the presence and absence of added cysteine and glutamic acid. Curve A, incubation mixtures contained Krebs-Ringer solution, rat red cells, cysteine 0.008 M, glutamic acid 0.008 M, and glycine; Curve B, the same as in Curve A, except that cysteine and glutamic acid were not added.

FIG. 2. Incorporation of labeled glycine and labeled glutamic acid into rat erythrocyte glutathione. Incubation mixtures contained added amino acids as follows: cysteine 0.006 M, glutamic acid 0.006 M, and glycine 0.0015 M.

By extrapolating the values in Curve B to zero glycine concentration, a limiting value of 6.8 μmoles per 100 ml. of cells is obtained for GSH formation during the 2.5 hour period. At this rate of formation, 50 per cent replacement of the non-labeled by the labeled glycine in GSH would require 37 hours in red cells with a GSH content of 200 μmoles per 100 ml. of cells. The time for 50 per cent replacement under aerobic conditions may be estimated to be approximately 55 hours, since the rate of aerobic formation was found to be about two-thirds of the anaerobic rate (Table II).

Incorporation of Glutamic Acid—In Fig. 2, the time-courses of glycine and glutamic acid are compared. After sufficient time had elapsed to

carry the reactions past the initial stage, the rates of incorporation were nearly equal. Johnston and Bloch (11) obtained similar results in a 1 hour incubation of these amino acids with extracts of pigeon liver, and interpreted their findings as suggesting total synthesis of GSH.

Different Levels of Endogenous GSH—A comparison was made of the rates of glycine incorporation into erythrocytes with two different levels of endogenous GSH. In studying the effect of GSH concentration, no way was found to vary the GSH content of the cells other than by dietary means since GSH does not penetrate the cells either *in vitro* or after its injection into the circulatory blood. Erythrocytes with a high level of GSH were obtained from the pooled blood of young rats which had been maintained for several weeks on a methionine-deficient diet (1). Those with a lower GSH content were from similar animals that had been kept on the same diet supplemented with 0.5 per cent of methionine. As shown in Fig. 3, there was no significant difference in GSH synthesis in the two groups of erythrocytes. While this finding would be expected in the event of total GSH synthesis, it could conceivably be produced as well by an enzyme-catalyzed exchange of labeled glycine with the glycyl group of GSH under the conditions existing in the erythrocyte. Because of the relatively high concentration of GSH in the cell, it is possible that some factor other than GSH concentration might determine the rate of reaction.

Nucleated Cells—Contrary to expectations, nucleated cells from duck blood produced a glycine incorporation of only about one-third of the incorporation found for normal rat erythrocytes (Fig. 4). It may be pointed out, incidentally, that a comparison of the absolute amounts of GSH formation in rat erythrocytes shown in Figs. 3 and 4 should take into account the difference in the levels of the glycine added to the incubation mixtures.

Induction Period—In certain other systems, it has been shown that the formation of GSH from the three constituent amino acids is accompanied by an induction period which can be eliminated by the addition of γ-glutamylcysteine to the reaction mixture (12, 13). In the present system, the initial portion of each time-course curve exhibits a similar induction period, but this was not altered by the addition of γ-glutamylcysteine to the suspension medium. (The γ-glutamylcysteine was kindly supplied by Dr. J. E. Snoke.) This failure of γ-glutamylcysteine to affect the reaction may be due to a lack of ability of the dipeptide to penetrate the red cell. Furthermore, in the present system, the initial stages may be complicated by the presence of endogenous γ-glutamylcysteine (14) and by a possible lag in the diffusion of amino acids into the cell.

Incubation in Plasma—In Table I, the effect of incubation in plasma and in mixtures of plasma with the Krebs-Ringer solution may be seen. At a
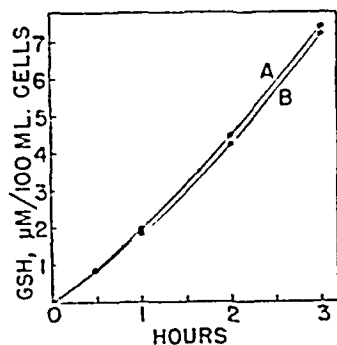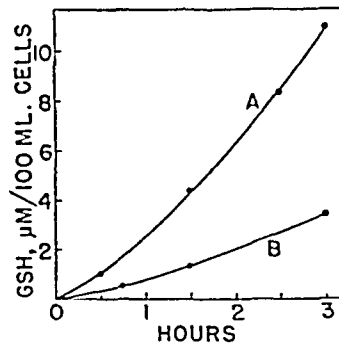
FIG. 3.   FIG. 4.

FIG. 3. Incorporation of labeled glycine into the glutathione of erythrocytes containing different concentrations of endogenous glutathione. Curve A, 280 μmoles per 100 ml. of cells; Curve B, 180 μmoles per 100 ml. Incubation mixtures contained Krebs-Ringer solution, rat red cells, and labeled glycine 0.00066 M.

FIG. 4. Comparison of rates of incorporation of labeled glycine into the glutathione of rat and duck erythrocytes. Curve A, rat cells; Curve B, duck cells. Incubation mixtures were the same as in Fig. 1, except that the concentration of labeled glycine was 0.0015 M.

TABLE I

Effect of Concentration of Plasma on Incorporation of Labeled Glycine into Erythrocyte Glutathione

Erythrocytes were incubated with labeled glycine in mixtures of plasma and Krebs-Ringer solution for 2.5 hours at 37° under 5 per cent carbon dioxide and 95 per cent nitrogen.

Volumes per cent plasma*	Volumes per cent Krebs-Ringer solution	C.p.m. per μmole glutathione†
Added glycine in plasma, 0.0112 M; in Krebs-Ringer solution, 0.0112 M		
100	0	5.9
67	33	7.2
33	67	7.7
0	100	9.0
Added glycine in plasma, 0.0008 M; in Krebs-Ringer solution, 0.0008 M		
100	0	107
67	33	103
33	67	111
0	100	101

* The figures represent the volume of plasma divided by the sum of the volumes of plasma and Krebs-Ringer solution and multiplied by 100.

† Each figure represents the specific activity of glutathione isolated from 2 ml. of the incubation mixture after the addition of 25 mg. of carrier glutathione.

high glycine level, the amount incorporated was less in plasma than in Krebs-Ringer solution. This result is in contrast to the accelerating effect of plasma on amino acid incorporation into reticulocyte proteins (15). In the present work, however, the effect of plasma disappeared at a low glycine concentration.

Effect of Oxygen—Four experiments were performed on rat erythrocytes to determine the amount of glycine incorporated under aerobic conditions. In each experiment the level of incorporation was substantially lowered by the presence of oxygen (Table II). That the GSH concentration was

TABLE II

Effect of Oxygen on Incorporation of Labeled Glycine into Erythrocyte Glutathione

Experiment No.	Incubation time	Glutathione formed*			Aerobic-anaerobic†	
		Anaerobic	16 per cent O₂	95 per cent O₂	16 per cent O₂	95 per cent O₂
	hrs.					
1	1	3.98	3.00	2.68	0.753	0.670
2	2	9.20	5.86	6.37	0.637	0.692
3	3	13.6	9.13	6.98	0.671	0.513
4	3	8.33	6.56	5.80	0.788	0.696
Average..					0.71	0.64

In Experiments 1 to 3, the incubation mixtures contained Krebs-Ringer solution, rat red cells, labeled glycine 0.0015 M, cysteine 0.006 M, and glutamic acid 0.006 M. In Experiment 4, cysteine and glutamic acid were omitted. Gas mixtures contained CO_2 5 per cent and N_2 as needed to make up the gas volume to 100 per cent.

  * Micromoles of glutathione formed from labeled glycine per 100 ml. of cells.

  † Ratios were calculated from the figures in the preceding columns.

not materially decreased during the incubation was demonstrated by determining GSH and non-protein sulfhydryl groups before and after incubation. A similar decrease in glycine incorporation was observed in duck erythrocytes under aerobic conditions, but the percentage change was less constant than in rat cells.

DISCUSSION

The results indicate that erythrocytes have the ability to incorporate glycine into GSH. Although total synthesis has not been conclusively demonstrated, the results are in harmony with the occurrence of total synthesis. The close correspondence between the rates at which glycine and glutamic acid are incorporated into GSH following the period of induction and the stimulating effect of added cysteine and glutamic acid

on GSH formation suggest at least a measure of total synthesis. This view is strengthened by the observation that the rate of GSH formation appears to be independent of the concentration of GSH in the erythrocyte.

SUMMARY

Glycine is rapidly incorporated into the glutathione of intact rat and duck erythrocytes *in vitro*. The simultaneous addition of cysteine and glutamic acid was found to increase the incorporation, and oxygen to decrease it. The incorporation rate was independent of the concentration of endogenous glutathione.

Addendum—Dimant, Landsberg, and London (16) have recently demonstrated that the incubation of human erythrocytes and of duck erythrocytes with glycine-2-C^{14} results in the incorporation of labeled carbon into the glutathione.

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THE TURNOVER OF ERYTHROCYTE
GLUTATHIONE IN THE RAT

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Several observations have been reported which suggest that the intact rat erythrocyte is able to synthesize and destroy glutathione (GSH). Anderson and Mosher (1) detected radioactivity in the erythrocyte GSH of rats following the administration of S^{35}-labeled cystine. In this laboratory it has been observed that C^{14}-labeled glycine is rapidly incorporated into the GSH of rat red cells *in vitro* (2). Furthermore, erythrocyte GSH levels have been found to undergo large changes as a result of adding and withdrawing methionine dietary supplements (3).

In view of these observations, it seemed of interest to investigate further the rate of GSH metabolism in the rat erythrocyte. In the present study the rate of disappearance from erythrocytes of previously labeled endogenous GSH was measured. At the same time, the distribution of radioactivity among the component amino acids was studied by chromatographic methods. The erythrocyte GSH was labeled by injecting labeled glycine.

EXPERIMENTAL

Male Sprague-Dawley rats were employed. The animals were provided with Purina laboratory chow and drinking water *ad libitum* throughout the experimental period. Each rat was injected intraperitoneally with a single dose of 14 μc. of glycine-2-C^{14} (Tracerlab) per 100 gm. of body weight. Blood samples were collected at intervals by heart puncture. Hematocrit values were determined by means of Van Allen tubes. Erythrocyte GSH was isolated as the cuprous mercaptide from 0.5 ml. aliquots of blood after deproteinization with 10 per cent trichloroacetic acid and the addition of 25 mg. of GSH to serve as carrier. The procedures used for isolating the GSH, and for preparing and counting the samples, were similar to those previously described (2). Counting times were sufficient to give a standard error of 5 per cent or less.

For determining the distribution of radioactivity among the component amino acids, GSH was hydrolyzed and the liberated amino acids were separated as 2,4-dinitrophenyl (DNP) derivatives by chromatographic adsorption on a silicic acid column. GSH cuprous mercaptide samples prepared from blood withdrawn at various intervals were first treated with hydrogen

sulfide to precipitate the copper. After centrifugation, the supernatant fluid obtained from each sample was boiled in 6 N HCl solution for 12 hours to hydrolyze the GSH. Since it had been found that the dinitrophenyl amino acids formed from the hydrolysate gave a double band on a silicic acid column corresponding to DNP-cystine and DNP-cysteine, a stream of oxygen was passed through the hydrolysate, after neutralization, to complete the conversion of cysteine to cystine. The hydrochloric acid was then evaporated, and the DNP derivatives of the amino acids were prepared as described by Sanger (4). The resulting reaction mixture was acidified and evaporated to near dryness to remove alcohol. The DNP derivatives were extracted from the residue with several portions of ether, and the combined extracts were washed with water. To prepare the DNP derivatives for chromatographing, the ether was evaporated and the derivatives were dissolved in 2 ml. of a 20 per cent solution of acetone in ligroin.

Preliminary experiments had shown that a mixture of DNP-cystine, DNP-glutamic acid, and DNP-glycine could be effectively separated on a silicic acid column with a developer containing 8 per cent acetic acid and 4 per cent acetone in ligroin. The column employed was similar to that used by Green and Kay (5), but contained silicic acid only and was not prewashed. The silicic acid was packed to a height of 150 mm. in a 12 × 300 mm. chromatographic tube of the type described by Zechmeister and Cholnoky (6). Before applying the sample, the column was wetted with a portion of the developer. Upon chromatographing the DNP derivatives with 50 ml. of the developer, three distinct bands at 5 to 17 mm., 26 to 40 mm., and 60 to 90 mm. were identified respectively as DNP-cystine, DNP-glutamic acid, and DNP-glycine. Identifications were made by comparing these derivatives with authentic specimens of the corresponding DNP-amino acids on separate and mixed chromatograms. Losses incurred in the reaction of 2,4-dinitrofluorobenzene with the amino acids, in column adsorption, and in the extractions were estimated by using authentic specimens of GSH and the amino acids. Corrections based on these losses were applied to the values reported for amino acid recoveries. Quantitative determinations of the amounts of DNP-amino acids in the column were made by cutting the bands, eluting with acetone, and reading the eluates in a Beckman model B spectrophotometer.

The acetone solutions containing the DNP-amino acid samples were evaporated to dryness on aluminum planchets under infra-red lamps, and the radioactivities determined. Corrections for self-absorption were made according to the data for wax (7).

Results

Disappearance of Labeled GSH—Exploratory work was carried out on four rats. After the injection of labeled glycine into these animals, the

radioactivity of the GSH rose rapidly, attaining a maximum in about 1 hour. For the next day or two the radioactivity remained relatively constant and then began a steady decline which approximated first order kinetics. The early rise in radioactivity apparently represented the period during which the labeled precursor was present in comparatively high concentration and was therefore undergoing rapid incorporation in the GSH of the cell.

With the time-course of labeled GSH formation and disappearance thus roughly determined, rats of the same age, weighing 215 to 235 gm., were
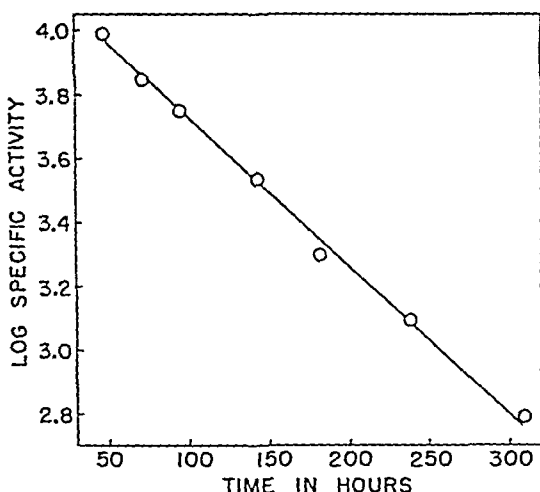
FIG. 1. Disappearance of labeled GSH from rat erythrocytes following a preliminary labeling of GSH with glycine-2-C^{14}. Specific activities are in counts per minute per millimole of GSH divided by the hematocrit, the GSH being that isolated from 0.5 ml. of blood after the addition of 25 mg. of carrier GSH. All points represent the averages of three animals, except those at 238 and 310 hours, which are for two animals only.

selected for the final experiment. Beginning at 48 hours after injection, blood samples were collected during a period of 262 hours. In Fig. 1 is the average disappearance curve for this interval. The half life, calculated from the curve, is 65 hours. Multiplying this figure by 1.44 gives the turnover time (average life) as 94 hours. A half life of the order of 4 days has recently been reported for the turnover of erythrocyte GSH in man (8).

Distribution of Radioactivity—Table I presents the distribution of radioactivity in the constituent amino acids of GSH at various times. It will be noted that relatively high isotope concentrations were found, as was to be expected, in the glycine component. Some radioactivity also appeared in the cysteine and the glutamic acid, the cysteine having almost twice the activity of the glutamic acid. The relative activities remained essentially

unaltered throughout the time of experiment, indicating that the three amino acids were lost at the same rate.

A typical analysis of labeled GSH formed *in vitro* is included in Table I for comparison. In contrast to the results obtained *in vivo*, no radioactivity could be detected in the cysteine and the glutamic acid components. Thus it appears that the red cell does not convert glycine to either one of these amino acids. The labeled cysteine and glutamic acid residues appearing in

TABLE I

Distribution of Radioactivity in Amino Acids of Erythrocyte GSH after Injection of Glycine-2-C¹⁴ in Rat

Time after injection	C.p.m. per mmole × 10⁻¹ in GSH*	C.p.m. per mmole × 10⁻¹ recovered in amino acids†	Per cent recovered activity‡	Per cent recovered activity in		
				Glycine	Cysteine	Glutamic acid
hrs.						
72	336	315.8	94	85.8	8.5	5.7
143	142	132.1	93	86.5	7.9	5.6
238	68	66.5	98	86.5	8.6	4.9
310	25	25.1	100	87.8	7.2	5.0
In vitro§	3394	3121	92	100	0.0	0.0

* Isolated from 0.5 ml. of blood after the addition of 25 mg. of carrier GSH.

† Sum of the activities of glycine, cysteine, and glutamic acid after corrections for losses incurred in the reaction of the amino acids with dinitrofluorobenzene, in column adsorption, and in the extraction of DPN derivatives.

‡ The recovery was calculated as the value in the third column divided by 1 per cent of the value in the second column.

§ Labeled by incubating a suspension of red cells with glycine-1-C¹⁴ for 2 hours at 37° under 5 per cent carbon dioxide and 95 per cent nitrogen.

red cell GSH must be formed from the injected glycine before the latter enters the cell.

DISCUSSION

The validity of the calculated turnover rate depends upon the assumption that the labeled glycine or other radioactive precursors of GSH formed from the injected glycine were not present in significant amounts during the time the rate of disappearance of the labeled GSH was being measured. Friedberg and Greenberg (9) found amino acids to be rapidly removed from the blood plasma, about 85 per cent disappearing within 15 minutes after administration.

Nevertheless, the possibility of an appreciable incorporation of labeled glycine returned to the amino acid pool from the breakdown of labeled

GSH and proteins cannot be excluded. Such incorporation would decrease the apparent turnover rate and increase the time. This effect, however, was probably not large, since the logarithmic radioactivity curve (Fig. 1) showed no tendency to level off during the period (four half lives) in which measurements were made.

That the disappearance of labeled GSH may occur as a result of enzyme-catalyzed exchange reactions seems unlikely in view of the constancy of the relative activities found in the amino acid components of the red cell GSH throughout the experiment. For the decline in labeled GSH to be brought about by an exchange of non-labeled amino acids for the labeled amino acid residues would require similar rates of exchange of all three amino acids. This condition seems improbable. Apparently exchanges of the amino acid residues in GSH do not occur in the red cell.

The results must be affected to some extent by the rate at which red cells disappear from the circulation. According to a recent report (10), rat erythrocytes undergo random physiological destruction with a 50 per cent survival of about 50 days. At this rate, cell destruction would be expected to decrease the apparent average life by no more than 3 or 4 per cent.

SUMMARY

The turnover of glutathione in erythrocytes has been studied by measuring the disappearance of labeled glutathione after a single injection of glycine-2-C^{14} into rats. The half life was calculated to be about 65 hours.

Radioactivity appeared in all three of the amino acid components of erythrocyte glutathione. The conversion of glycine to cysteine and glutamic acid occurs prior to the entrance of glycine into the cell.

The distribution of radioactivity among the amino acid residues remained unaltered throughout the time of experiment. This observation was interpreted as indicating the probable absence of exchanges of amino acids for the amino acid residues of glutathione in the red cells.

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TURNOVER OF PALMITIC, STEARIC, AND UNSATURATED FATTY ACIDS IN RAT LIVER*

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The advent of isotopes as a biochemical tool has given rise to considerable work designed to estimate the rate of metabolism of liver lipides in intact animals (1–9). In most instances the tracer dose was given for an extended period, and the estimates of the half turnover time were obtained from the time required for the tracer dose to reach half maximal concentration or the time required for the tracer concentration to fall to half maximal concentration after administration of the tracer was discontinued. The interval between measurements for these experiments was generally 24 hours; consequently, estimations of half turnover times for much less than a day were not obtained. Recently Hevesy et al. (7, 8) and Beeckmanns and de Elliot (10) have shown by means of the single dose technique the existence of a rapidly metabolizing liver lipide fraction in mice and rats. Although some workers have separated the saturated and unsaturated fatty acids (6, 8), most observations were made with the total fatty acids of the liver. Thus, there is little information on the rate of metabolism of one fatty acid relative to another. In the report that follows, specific activity-time curves for palmitic, stearic, and the unsaturated fatty acids of phosphatides and neutral fat of rat liver are presented.

EXPERIMENTAL

Weanling male rats of the Holtzman strain were reared in individual cages and were fed a fat-free purified diet consisting of the following parts: sucrose 76, "vitamin test" casein 20, Salts IV (11) 4, and L-cystine 0.2. To each 100 gm. of ration the following vitamins were added: thiamine hydrochloride 0.2 mg., riboflavin 0.3 mg., pyridoxine hydrochloride 0.25 mg., nicotinic acid 1.0 mg., calcium pantothenate 2.0 mg., choline chloride 100 mg., menadione 0.5 mg., folacin 0.2 mg., inositol 10 mg., biotin 0.01 mg., and vitamin B_{12} 0.005 mg. The fat-soluble vitamins were dissolved in methyl laurate, and each rat received 20 γ of vitamin A acetate, 0.04 γ of

vitamin D_2, and 1 mg. of α-tocopherol acetate orally at weekly intervals. Tetrabromostearic acid (m.p. 114–116°) methylated and then debrominated by zinc was used to supply the essential fatty acid requirement. Each rat received 90 mg. of methyl linoleate per day. At the end of 21 weeks, the rats had attained an average weight of 317 gm. Since the growth curve had essentially reached a plateau at this weight, the animals were judged to be in a steady state.

Each rat was then given 10 μc. of carboxyl-labeled sodium acetate (Tracerlab), having a specific activity of 1 μc. per μmole, by intraperitoneal injection. Animals were sacrificed, by decapitation, at 4 hour intervals beginning at 2 hours and extending to 44 hours after the acetate injection. The livers were removed and extracted twice with a hot 3:1 alcohol-ether mixture, and then by ether to obtain the lipides. The extracts were combined and then dried *in vacuo* in an atmosphere of nitrogen. The phosphatide and the neutral fat fractions were separated by acetone and magnesium chloride precipitation as described by Pihl and Bloch (5). Both fractions were saponified for 1 hour with 10 per cent boiling ethanolic potassium hydroxide in an atmosphere of nitrogen. After extraction of the fatty acids, the unsaturated fatty acids were oxidized to their polyhydroxy derivatives by cold alkaline permanganate according to the procedure of Nunn and Smedley-MacLean (12). The mixture of the saturated and the polyhydroxy acids was removed from the oxidation medium by ether extraction after acidification and solution of the manganese dioxide by sulfur dioxide. After evaporation of the solvent, the fatty acids were dissolved in 80 per cent aqueous acetone; the palmitic, stearic, and unsaturated fatty acids (as the polyhydroxy derivatives) were separated by the reverse phase chromatographic procedure of Howard and Martin (13). An aqueous solution of the potassium salt of each saturated acid was washed with petroleum ether (Skellysolve F) and the fatty acid extracted with this solvent after acidification. The petroleum ether-fatty acid solution was exhaustively washed with water, and the ether was removed by evaporation at 90°. The polyhydroxy acids were purified by copper lime precipitation (14) and the precipitate thoroughly washed with water. Each fatty acid sample was then oxidized (15) and counted, as barium carbonate, in a windowless flow counter. The counts were corrected for self-absorption to infinite thinness. Specific activity is expressed as counts per minute per mg. of barium carbonate.

RESULTS AND DISCUSSION

The specific activity-time curves of the phosphatide and neutral fat palmitic, stearic, and unsaturated acids (Figs. 1 to 3) show that the half turnover time of each of these acids is in the order of a few hours rather

than 1 day. The time at which maximal specific activity is reached agrees with the data of Hevesy (8) on the combined neutral fat fatty acids and the results that Gidez and Karnovsky (16) obtained with glycerol.

The second peak in the specific activity-time curves is clearly evident for all acids except neutral fat palmitic acid. Beeckmanns and de Elliot (10) have observed a similar event in the specific activity-time curves of liver lipides in the mouse. They concluded that the first peak represents the turnover of a minor lipide fraction. Hevesy and coworkers (7, 8) concurred in this conclusion. This interpretation of the rapid metabolism observed is not at variance with the turnover values of from less than 1 or 1.9 days

FIG. 1. Specific activity-time curves of palmitic acid of liver phosphatides and neutral fat following the injection of carboxyl-labeled acetate.

obtained from the long time constant dose experiments (4, 6). There has been no direct evidence, however, that the rapidly renewable fraction is a minor one. Since the fatty acids of both the liver phosphatides and neutral fat as well as glycerol of these two fractions (16) are rapidly renewable, distinguishing the presence of a minor fraction from the mass of liver lipides is not possible. Therefore, an alternative interpretation is that the mass of liver lipides is rapidly metabolized.

The second peak in the specific activity-time curves shown in Figs. 1, 2, and 3 could be the result of recycling of radiocarbon into the hepatic fatty acids from some other metabolite previously derived from the injected acetate. That an extensive number of non-fatty acid metabolites may arise from carboxyl-labeled acetate has been demonstrated by Katz and Chaikoff (17). It is conceivable that the second peak could arise, at least in part, from any of these compounds as well as from the extrahepatic fatty

acids. The second peaks could also be the result of differences in the rates at which the various components of either the phosphatide or of the neutral fat mixtures are metabolized. Differences in the rates of metabolism of the same type of lipide in the various parts of the cells might also account for the second peaks. However, the similarity between the specific activity-

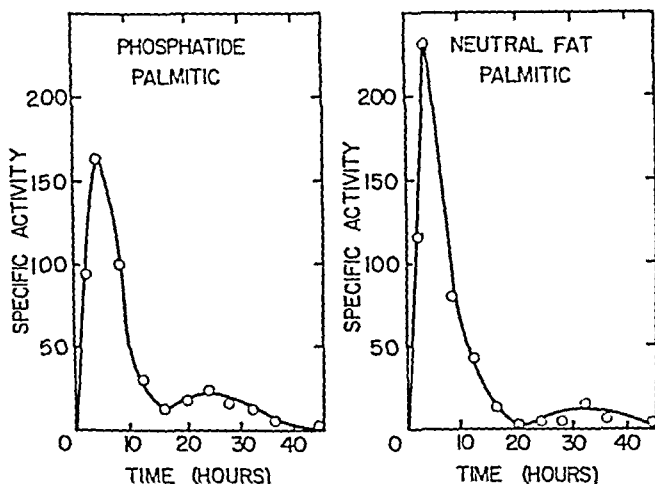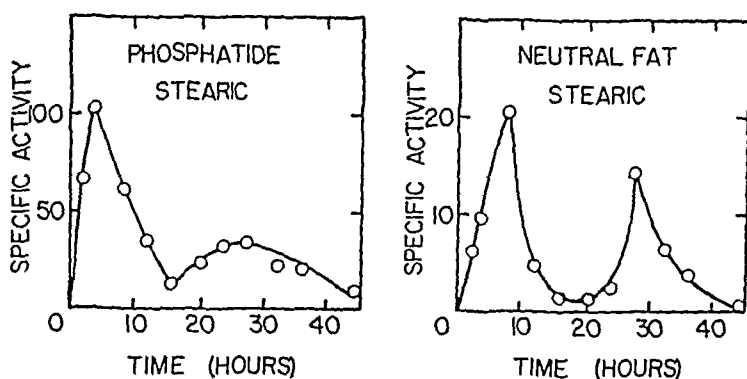
FIG. 2. Specific activity-time curves of stearic acid of liver phosphatides and neutral fat following the injection of carboxyl-labeled acetate.
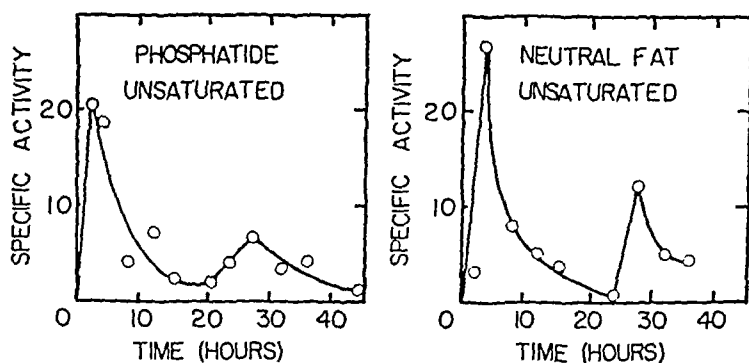
FIG. 3. Specific activity-time curves of the unsaturated fatty acids of liver phosphatides and neutral fat following the injection of carboxyl-labeled acetate.

time curves of the phosphatides and of the neutral fat would tend to render the latter two hypotheses less likely.

The points comprising the specific activity-time curves of the constant dose type of experiment were not taken at intervals frequent enough to permit observation of a rapid metabolism (4, 6, 8). Moreover, if points of the specific activity-time curves presented in this paper are selected at intervals approximating 24 hours, the half turnover times calculated from these points are in good agreement with values previously reported. Therefore, the turnover curves derived from measurements taken at daily intervals could represent the sum of a series of recycling events and thus yield half turnover times which were considerably longer than the true values.

Inspection of the specific activity-time curves for palmitic acid (Fig. 1) and the unsaturated fatty acids (Fig. 3) reveals no apparent difference between the metabolic rates of liver phosphatides and of liver neutral fat. The data presented for these acids support the conclusion of Pihl and Bloch (5) that the liver phosphatides are not obligatory intermediates in the synthesis of neutral fat. It is also apparent from an inspection of the curves in Figs. 1 to 3 that the metabolic rates of palmitic acid, the unsaturated acids, and phosphatide stearic acid are similar. In contrast, the specific activity of the neutral fat stearic acid rises more slowly than that observed for the other acids; hence it would appear that the metabolism of neutral fat stearic acid is slower than that of the other acids. Since the turnover of the phosphatide stearic acid is faster than that of the neutral fat stearic acid, it would seem likely that the rate of formation of

TABLE I

Average Fatty Acid Concentration in Rat Liver

Fatty acid	Concentration, mg. per 100 gm. liver*	
	Phosphatide	Neutral fat
Palmitic ..	66	45
Stearic..	119	21
Unsaturated	233	136

* The average liver weight was 9.4 gm.

the stearate ester bonds in the neutral fat fraction is slower than that of the other ester bonds in the liver lipides. Of course this hypothesis is based on the conclusion that the phosphatides are not obligatory intermediates of the neutral fat.

The specific activity values of the neutral fat palmitic acid are about the same as those of the phosphatide palmitic acid. Similarly, the unsaturated fatty acids of the neutral fat and the phosphatide fractions have approximately equal specific activity. In contrast, however, the specific activities of the neutral fat stearic acid are only about one-eighth those of the phosphatide stearic acid. Since both the neutral fat and phosphatide fractions are mixtures of several components, it is possible that one component especially rich in stearic acid does not attain rapid equilibrium with the remainder of the system. There is, however, no evidence at present to support such a supposition.

The results reported here confirm the observations of Pihl, Bloch, and Anker (6) and Hevesy (8) that the specific activity of the unsaturated fatty acids is less than that of the saturated fatty acids. These workers cal-

culated that the half turnover time of the unsaturated acids was about twice as long as that of the saturated acids. In contrast, however, the results of the experiment in this report indicate the half turnover times of the unsaturated fatty acids to be about the same as those of the saturated fatty acids, with the exception of neutral fat stearic acid.

The concentrations of the various fatty acids isolated are reported in Table I. The values were determined from the amounts isolated chromatographically; the unsaturated fatty acids are calculated as dihydroxystearic acid. It is apparent that, with the exception of neutral fat stearic acid, neither the specific activity level nor the turnover of the fatty acids was correlated with the hepatic concentration.

SUMMARY

Two distinct peaks have been observed in the specific activity-time curves for the individual fatty acids of the phosphatide and neutral fat fractions of rat liver. It is apparent from these curves that the turnover of hepatic fatty acids is faster than was previously reported. There were no differences in the rates of metabolism of the various phosphatide fatty acids, neutral fat palmitic acid, or neutral fat unsaturated acids. However, the rate of metabolism of neutral fat stearic acid appeared to be slower than that of the other fatty acids. In the case of palmitic acid and the unsaturated fatty acids, the specific activity values of the phosphatide acids were similar to those of the neutral fat acids. In contrast, however, the specific activity values of the phosphatide stearic acid were about 8 times those of the neutral fat stearic acid.

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STUDIES IN HISTOCHEMISTRY

XXXVIII. DETERMINATION OF SUCCINIC DEHYDROGENASE IN MICROGRAM AMOUNTS OF TISSUE AND ITS DISTRIBUTION IN RAT ADRENAL*

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A considerable literature has developed on the use of tetrazolium compounds for localization of succinic dehydrogenase activity in tissues by staining reactions. However, there are relatively few reports on the use of these compounds for macroquantitative analysis of this enzyme (1–5). In the present report, a quantitative method applicable to microgram amounts of tissue, such as microtome sections, will be described. The application of this method to a study of the histological distribution of the enzyme in the adrenal gland of the rat will also be given.

The quantitative tetrazolium methods are based on the reduction of tetrazolium compounds to water-insoluble colored formazans, when the former act as hydrogen acceptors during the enzymatic oxidation of succinate to fumarate. The formazan is extracted with a fat solvent and this solution is subjected to colorimetry. Advantages in the use of neotetrazolium over triphenyltetrazolium salts for quantitative work have been pointed out by Glock and Jensen (5); neotetrazolium was used in the present investigation. After difficulties encountered in the elaboration of the micromethod were resolved, a procedure was developed which proved to be relatively simple and rapid, as well as sensitive and accurate.

EXPERIMENTAL

Apparatus—The reaction vessels were 25 × 4 mm. Pyrex glass tubes, of 0.3 ml. capacity, fitted with flat top ground glass stoppers. The tubes and stoppers were marked to facilitate matching. In addition to the ground glass stoppers, a set of stoppers was made from short lengths of plastic tubing plugged at one end with glass rod ((6) p. 169). Constriction pipettes ((6) p. 172) of the following capacities were employed: 5.0, 11.6, 17.7, and 31.2 μl. All of the pipettes, except the largest which was used for tetrachloroethylene, were treated with silicone to avoid creeping

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of liquid by filling them with a solution of Drifilm (General Electric Company). They were then drained, dried, and rinsed several times with distilled water. An additional untreated, roughly calibrated constricted pipette of 15 to 17 μl. capacity was used to fill the cuvettes. The narrow stem of this pipette had an outer diameter of <1 mm. so that its tip could fit to the bottom of the lumen of the cuvette, and a calcium chloride drying tube was attached to the pipette to prevent uptake of moisture from the breath. Calcium chloride tubes were used similarly with the 17.7 and 31.2 μl. pipettes.

Colorimetry was carried out in glass capillary cuvettes with a 1 mm. lumen diameter, 10 mm. in length, and 7.9 μl. capacity. The cuvettes were used with a microscope colorimeter (7) employing an interference filter (Farrand) having peak transmittance at 519 mμ. Other apparatus used, including the "buzz" mixer and micro centrifuge (Microchemical Specialties Company), was the same as that employed in previous work (8).

Reagents—The following solutions were prepared as indicated: M/15 phosphate buffer, pH 7.7; 0.068 M disodium succinate in M/15 phosphate buffer, pH 7.7; 0.5 per cent neotetrazolium chloride (Dajac) (to 5 ml. of distilled water and 0.05 ml. of 1 N HCl in a 10 ml. volumetric flask add 50 mg. of the dye, stopper, and shake under running warm water until all dissolves, cool to room temperature, make up to volume, centrifuge to sediment any particles, and then store the supernatant solution in the dark); 5.34 $\times$ 10^{-4} M disodium ethylenediaminetetraacetate (Versenate, Bersworth) in M/15 phosphate buffer, pH 7.7; alcohol-tetrachloroethylene mixtures (1:1 and 2:1 by volume of absolute ethyl alcohol and tetrachloroethylene (Eastman Kodak)); stock standard solution (dissolve 2.3 mg. of neotetrazolium diformazan (Synthetical Laboratories) in 25 ml. of alcohol-tetrachloroethylene mixture 1:1); working standard solutions (dilute stock standard with the 1:1 mixture 2.5, 3.0, 5.0, 10.0, 25.0, and 50.0 times and store all standard solutions in the cold and dark).

Preparation of Samples for Chemical Analysis and Histological Control— Rat liver homogenates were prepared by passing chilled fresh liver through a stainless steel tissue press having 1 mm. pores, then homogenizing with 9 volumes of M/15 phosphate buffer, pH 7.7, in a Potter-Elvehjem apparatus cooled in an ice bath. Dilutions were made with the Versenate-phosphate buffer to give the desired concentration in any experiment.

When microtome sections of fresh frozen rat adrenal tissue were used for analysis, samples were prepared as described by Bahn and Glick (9), by using a punch 1.5 mm. in diameter and cutting 16 μ sections. Adjacent serial sections were used in the following order: one section for histology, two for enzyme analysis, two for analytical control, and one for protein nitrogen determination. The sections taken for histological ex-

amination were treated and stained with toluidine blue by the procedure of Bahn and Glick (9), and the protein nitrogen analysis was performed by the method of Nayyar and Glick (10).

Procedure for Succinic Dehydrogenase Determination—(1) Pipette 11.6 μl. of the Versenate solution into the bottom of each reaction vessel. (2) Add 5 μl. of the neotetrazolium solution to the liquid in each vessel, close with glass stoppers, and place at $-20°$ to freeze. (3) With the tip of a drawn out glass rod, place a frozen microtome section of tissue or other frozen sample on the frozen liquid and restopper. The following steps through (5) must be carried out in dim light. (4) Hold each vessel between the fingers until half of the liquid has melted, add 5 μl. of buffered substrate solution, mix briefly by "buzzing," but do not break up the tissue any more than necessary. Seal with plastic stoppers, and suspend in a thermostat at 37°. For this step allow 2 to 3 minutes for each tube and record the time at which each is placed in the thermostat. (5) Allow the contents of the tube to digest for 1 hour, centrifuge for 0.5 to 1 minute at 3800 $\times$ *g*, draw off the supernatant fluid as completely as possible, and discard. The 17.7 μl. pipette can be used for this purpose. Pipette 31.2 μl. of alcohol-tetrachloroethylene mixture (1:1) into each vessel, "buzz," and seal with glass stoppers. The 2 to 3 minute interval following removal of each tube from the thermostat should be sufficient to permit all of the operations in this step. (6) Add to all reaction vessels 17.7 μl. of absolute alcohol and mix. (7) Close the vessels with their respective glass stoppers, and centrifuge for 5 minutes at 1900 $\times$ *g*. The color of the liquid is stable for a number of hours. (8) Set the microscope photometer to 100 per cent transmission with the alcohol-tetrachloroethylene mixture (2:1). For each sample fill a cuvette and immediately measure the absorbancy at 519 mμ. (9) Add 17.7 μl. of absolute alcohol to 31.2 μl. of a working standard solution and use this mixture to check the performance of the instrument by comparing it with the calibration curve. (10) Correct the absorbancy of the unknown for the no substrate blank and express enzyme activity as the amount of formazan produced per hour. (A no enzyme blank prepared with the complete reaction mixture, but without the biological sample, gave a zero absorbancy and therefore could be ignored. However, no substrate blanks containing all reaction components except succinate, which was replaced by phosphate buffer, were appreciable and had to be included.)

Reduction of Neotetrazolium—Common practice for the chemical reduction of a tetrazolium salt has been the addition of a few crystals of sodium hydrosulfite to the solution. Nordmann *et al.* (2) proposed the use of ammonium sulfide as the reducing agent and pointed out that direct hydrogenation could be employed. In this study hydrosulfite was used in

controlled quantities, both as the solid and as a freshly prepared solution which is stable for 1 to 2 minutes. Reproducibility of the formazan solution could not be obtained. In view of the fact that hydrosulfite undergoes simultaneous oxidation and reduction in aqueous solution according to the reaction $2Na_2S_2O_4 \rightarrow Na_2S_2O_3 + Na_2S_2O_5$, the fresh solution was used with an equivalent of added ascorbic acid in an attempt to suppress the oxidizing action of the $Na_2S_2O_5$, without success. The procedure of Glock and Jensen (5), in which solid hydrosulfite is used to produce a diformazan suspensoid from which serial dilutions can be made, also yielded irregular results.

Other reducing agents tested were arsenite, thiosulfate, sulfite, hydrogen sulfide, cyanide, ascorbic acid, β-mercaptothiazoline, metallic sodium, and ammonium sulfide. Only the last two yielded formazan, and the ammonium sulfide was the better, giving a highly reproducible formazan solution which followed Beer's law when measured at 550 mμ. However, the absorption curve for this formazan solution was too different from that obtained by enzymatic reduction to make its use desirable for colorimetric standardization (Fig. 1).

From Fig. 1 it is apparent that both the diformazan and the enzymatically reduced product have a common maximal absorption at 520 to 530 mμ. Solutions of the diformazan in tetrachloroethylene-alcohol followed Beer's law over the absorbancy (D) range of 0.05 to 0.60. The slope of this linear relationship was as follows: millimicrograms of neotetrazolium diformazan = 1104 D. For absorbancies from 0.60 to 0.90 the values were less than would be expected from the linear relationship.

Extraction of Diformazan—Solvents such as acetone and ethyl acetate, that have been used to extract formazans from tissues or homogenates, are too volatile for use in the capillary cuvettes employed in this work. Another difficulty has been incomplete extraction from wet tissue samples with any of the solvents which have been employed. *n*-Butanol, *n*-propanol, and tetrachloroethylene were tested in the present study, and of these the last proved to be most effective. Difficulty was still experienced in getting complete extraction, owing to binding of the diformazan to protein, and treatment with acids, bases, or bile salts was ineffective. The problem was finally solved by addition of an equal volume of absolute alcohol to the tetrachloroethylene. The alcohol served to dehydrate and denature the protein, rendering the diformazan easily extractable. The extraction was applied most effectively by centrifuging the diformazan-tissue mixture, pipetting off the supernatant fluid, and then extracting the residue with the alcohol-tetrachloroethylene solution. While the 1:1 proportion was preferable for tissue sections, a 3:1 mixture of alcohol-tetrachloroethylene proved desirable for liver homogenates.

Stopping Enzyme Action—Methods for stopping the enzyme action at the end of the digestion period were tested. At first, immersion of reaction tubes and blanks in boiling water for periods from 10 seconds to 1 minute was tried. This caused an increase in absorbancy of about 30 per cent. The use of the alcohol-tetrachloroethylene for extraction of formazan proved to be an adequate means of blocking further enzyme action.

Optimal Conditions—The procedure given earlier in this paper was modified as follows for use with liver homogenate to permit study of optimal
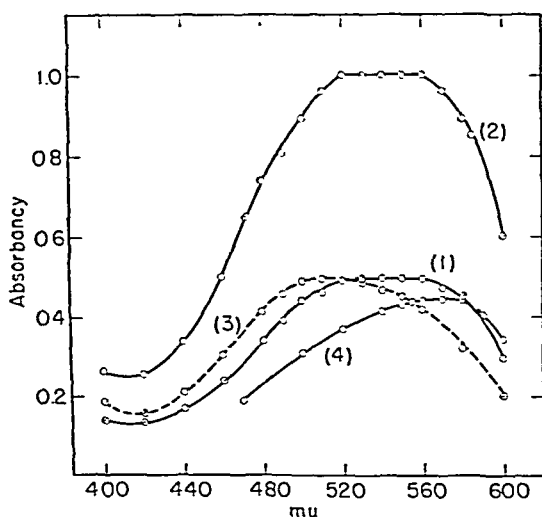
FIG. 1. Absorption curves of neotetrazolium formazan obtained by different reducing agents. Curve 1, neotetrazolium diformazan standard 1.15 mg. per cent; Curve 2, neotetrazolium diformazan standard 2.3 mg. per cent; Curve 3, enzymatic reduction; Curve 4, ammonium sulfide reduction.

conditions for assay: 5 μl. of neotetrazolium chloride and 5 μl. of succinate solutions were placed in a reaction tube. 11.6 μl. of diluted homogenate were added, and the tube was "buzzed" to mix, stoppered, and placed in a thermostat at 37° for digestion (1 to 3 hours). Then the earlier procedure was continued, starting with step (5), but 60 μl. of 3:1 alcohol-tetrachloroethylene were used in step (5), step (6) was omitted, and then steps (7 to 10) were followed.

In a control experiment the loss of formazan color during digestion and up to the time of extraction, owing to the room illumination, was found to vary from 7 to 14 per cent. Therefore, all digestions were conducted in the dark, and the steps immediately following up to the extraction were carried out in subdued light. Once the formazan was extracted with the alcohol-tetrachloroethylene, the color was stable.

Various substances were added to the reaction mixture in an attempt to enhance the reaction. Gum ghatti was employed to stabilize the neotetrazolium by preventing flocculation in the alkaline reaction medium. However, a 30 per cent loss in activity resulted, and its use was discontinued (Table I).

Kun and Abood (1) and Zöllner and Rothemund (11) found that sodium cyanide in the reaction mixture did not affect the enzyme activity when

TABLE I

Effect of Additions on Succinic Dehydrogenase Activity

Final concentration, gum ghatti 0.05 per cent, Versene 2.13×10^{-4} M, NaCN 1×10^{-3} M, $CaCl_2$ 4×10^{-3} M, $AlCl_3$ 4×10^{-3} M, sodium succinate 1.7×10^{-2} M, neotetrazolium 0.125 per cent, total phosphate 5×10^{-2} M.

Sample	No. of analyses	Absorbancy, mean ± standard error	Per cent change
Control	5	0.76 ± 0.022	
Gum ghatti	5	0.53 ± 0.025	−30
Control	3	0.39 ± 0.019	
NaCN	3	0.36 ± 0.012	−7
Control	2	0.53 ± 0.005	
Versene	3	0.90 ± 0.019	+70
" NaCN	4	0.88 ± 0.024	−2
" Ca^{++}, Al^{+++}	2	0.85 ± 0.005	−6
Control	3	0.60 ± 0.020	
Ca^{++}, Al^{+++}	3	0.60 ± 0.029	0
Versene	2	0.73 ± 0.000	+22
" Ca^{++}, Al^{+++}	3	0.80 ± 0.037	+9
Control	4	0.52 ± 0.010	
Versene	4	0.68 ± 0.015	+31
Ca^{++}, Al^{+++}	4	0.54 ± 0.035	+4

triphenyltetrazolium was employed aerobically. Activation by cyanide of the reduction of triphenyltetrazolium by yeast cell suspensions was reported by Nickerson and Merkel (12). A 50 per cent inhibition by cyanide of the enzymatic reduction of blue tetrazolium under aerobic conditions was reported by Seligman and Rutenberg (13), but no inhibition was found anaerobically. From Table I it is clear that under the conditions used cyanide had little effect.

In staining reactions it has been reported that calcium and aluminum ions activate the enzyme (14, 15), but under the conditions of this work no favorable effect was obtained (Table I).

Versenate was found to exert a constant activation from 2×10^{-3} to 2×10^{-5} M (Table I). This is in accord with the well known fact that heavy metal ions inhibit the activity by sulfhydryl binding and that chelating agents of these ions remove the inhibition. Kun (16) used histidine to chelate heavy metal contaminants. Nickerson and Merkel (12) reported the increased reduction of triphenyltetrazolium by yeast cells in the presence of Versenate.

No further increase in activity was observed when the neotetrazolium was used in final concentrations of from 0.05 to 0.20 per cent. Maximal activity was obtained with 0.017 M sodium succinate, increases to 0.04 M did not change the activity, but decreases to 0.004 M caused a small drop in activity. Potter and Schneider (17) recommended the use of 0.017 M succinate for the enzyme assay by the oxygen consumption method.

Duplicate or triplicate analyses with liver homogenate at final concen-

TABLE II

Reproducibility of Succinic Dehydrogenase Determination in Rat Liver

Sample	No. of analyses	Absorbancy		
		Mean	Optimal estimate	Standard error
Homogenate (0.116 mg. tissue)..	8	0.49	0.028	0.011
Tissue section (2 mm. diameter, 16 μ thick, 0.05 mg.)	4	0.50	0.024	0.012

trations of 0.29, 0.58, 0.87, and 1.16 per cent, corresponding to 0.058, 0.116, 0.174, and 0.232 mg. of fresh liver per reaction vessel, were conducted for a digestion period of 2.5 hours. The slope of the activity-concentration curve was constant up to a concentration of 0.58 per cent, but at higher concentrations it decreased.

Fairly constant activity was observed over the range pH 7.4 to 7.8. A 20 per cent loss was noted at pH 7.2. The pH of the digestion medium finally employed was 7.6.

A linear relationship between enzyme activity and time was obtained for digestion intervals of 0.5, 1, 2, 3, and 4 hours with 0.58 per cent liver homogenate.

Reproducibility—Analyses on liver homogenates and tissue sections were carried out to evaluate the reproducibility of the method. The variation, expressed as the optimal estimate of the mean, was about 5 per cent in both cases (Table II).

The enzyme activity of a volume of blood (0.056 μl.) equal to the volume of two sections, 16 μ thick and 1.5 mm. in diameter, was determined in the

presence and absence of added succinate.   The absorbancy of the slightly colored alcohol-tetrachloroethylene extract was found to be the same in both cases, indicating that the blood in these tissue samples does not contribute significantly to the enzymatic reduction of neotetrazolium.   Sections of tissue kept at −20° for 24 hours retained all of their enzyme activity.   Frozen liver stored at −20° for 9 days did not lose activity.

RESULTS AND DISCUSSION

Fifteen determinations of the succinic dehydrogenase activity of single fresh frozen microtome sections (2 mm. in diameter, 16 μ thick, 0.05 mg.) from the livers of six rats gave a mean value of 394 mμgm. of formazan per hour per section, with an optimal estimate of the mean of 54.6 and a standard error of 22.3.   Corresponding data for the mean protein N were 1.445 γ per section, optimal estimate 0.265, and standard error 0.108.   Thus the mean activity, expressed as millimicrograms of formazan per hour per microgram of protein N, was 272.

The distribution of succinic dehydrogenase in the left rat adrenal is shown in Fig. 2; it is apparent that the activity in the reticularis was as great as that in liver.   It should be noted that the no substrate blank became particularly important in the adrenal medulla.   In liver the blank was 10 per cent of the total absorbancy for 1 hour digestions.

Qualitative distributions of the enzyme in fresh frozen sections of the rat adrenal, as indicated by staining reactions, have been reported, and the results appear to be conflicting.   Thus, Zweifach et al. (18) observed no particular localization in the cortex with triphenyltetrazolium, the formazan being uniformly deposited over all of the cortical regions.   In the absence of added succinate, endogenous substrate gave a formazan deposition in all of these zones except for an area at the juncture of the glomerulosa and fasciculata.   Rutenberg et al. (15) could detect no formazan product in any adrenal zone when blue tetrazolium was used with added succinate either aerobically or anaerobically.   The multiplicity of the variables, many of which cannot be adequately controlled, may account for the differences in the staining reactions.   A reliable quantitative method with proper controls, such as that outlined in this communication, could be used to check the validity of the localizations obtained by staining procedures, at least in certain organs such as the adrenal.

After this investigation was completed, a paper by Defendi and Pearson (19) appeared in which a quantitative method for succinic dehydrogenase was described, based on the treatment of tissue sections with 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride to give formazan staining.   The stain was extracted with ethyl acetate and subjected to

colorimetry. In an earlier report (20) these authors stated that the stain for the enzyme activity showed localization in the glomerular zone of the rat adrenal, but that the presence of lipide in the other zones interfered with the staining reaction. No such interference was found with the method employed in this communication, and the interpretation of the data on the adrenal presented no difficulty.
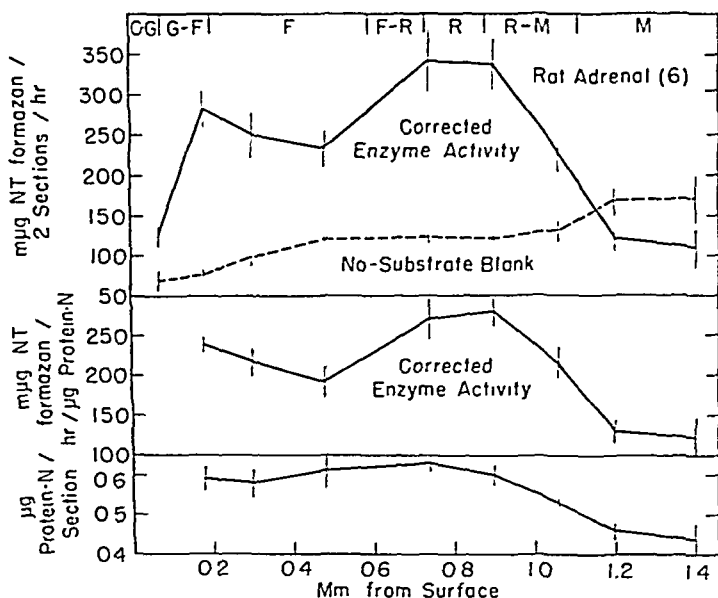
FIG. 2. Distribution of succinic dehydrogenase activity in the left adrenal of the male albino rat (Holtzman). Microtome sections 1.5 mm. in diameter, 16 μ thick, volume 0.028 μl. The points represent means; the vertical lines through points, the standard errors of the means. Histological zones are indicated by C (capsule), G (glomerulosa), F (fasciculata), R (reticularis), M (medulla). When sections are mixtures of two zones, both letters are used. The composite curves represent results from six adrenals, indicated by the number in parentheses.

SUMMARY

1. A colorimetric method for the quantitative determination of succinic dehydrogenase in microgram samples of tissue was described which is based on the use of neotetrazolium as the hydrogen acceptor.

2. The use of an alcohol-tetrachloroethylene mixture for the extraction of the formazan product, and Versenate as an activator of the enzyme, increased the precision and sensitivity of the method.

3. The activity of the enzyme in rat liver and its quantitative distribution in the rat adrenal were measured.

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THE PHENOLIC ACIDS OF HUMAN URINE*

PAPER CHROMATOGRAPHY OF PHENOLIC ACIDS

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In the course of an investigation of the metabolism of aromatic compounds in phenylketonuria, it was found that, along with the abnormal metabolism of phenylalanine in this disease, there are other striking deviations from normal. These are manifested by the excretion of greatly increased amounts of some indole acids (1) and phenolic acids (2, 3) which are present in normal human urine in small quantities. In order to gain a clearer understanding of the deranged metabolism of aromatic compounds in phenylketonuria and in other disorders, it became necessary to examine the phenolic constituents of normal human urine.

Some procedures already have been reported for the chromatographic characterization of various phenolic acids (4–9). The compounds for which detailed information has been reported are few in number, and there has been no systematic investigation of the simple compounds which might be expected to occur in urine. The purpose of this paper is to report a description of the phenolic acids of human urine. A combination of solvent systems which is suitable for the separation of forty-nine authentic aromatic acids by two-dimensional paper chromatography has been developed.

EXPERIMENTAL

Some of the compounds in these experiments were obtained from commercial sources. Others were prepared by methods recorded in the literature or were made by unequivocal syntheses when they had not been reported previously. Commercially available solvents of the best grade which could be purchased were used without further purification.

5 γ samples of the compounds were satisfactory for testing their behavior on chromatograms; larger amounts did not show significantly different behavior in the solvent systems reported here (Table I). When chromatograms were developed with benzene-propionic acid-water, the paper sheets

were allowed to equilibrate in the chambers for 1 hour before development was commenced. No preliminary equilibration was employed with the other solvent systems. Fresh solvents were used for each chromatogram. This is critical with the benzene-propionic acid-water system, since enough of the water in the mixture is absorbed preferentially by paper to change the composition of the mixture and to cause a marked disturbance of the movement of compounds in the system if the solvent is used a second time. The n-butanol-acetic acid-water system was prepared freshly for each run, as suggested by Berry et al. (10); R_F values were not affected markedly if the solvent was allowed to age, however. It was necessary to apply weights to the covers of chambers containing the isopropyl alcohol-ammonia-water system; otherwise sufficient ammonia escaped during an overnight run to cause variations in R_F.[1]

Chromatograms were developed at 25° by capillary ascent on Whatman No. 1 filter paper; the solvent fronts were allowed to advance 20 to 25 cm. Chromatograms were examined under ultraviolet light (Mineralight) for the ability of compounds to fluoresce or to quench the background fluorescence of the paper. The phenolic acids were visualized by coupling them with diazotized sulfanilic acid as described by Berry et al. (10) and with diazotized p-nitroaniline (4). Reducing phenols and aromatic keto acids were detected with ammoniacal silver nitrate (10), and aroylglycines with the hippuric acid reagent of Gaffney et al. (11). The Folin-Ciocalteu reagent (12) could be used to detect phenolic acids, but the lack of a characteristic color with individual compounds made this reagent of little use in comparison with the diazotized amines.

When chromatograms have been developed in the benzene-propionic acid or butanol-acetic acid system they should be allowed to stand overnight or should be heated in an oven at 100° for 3 minutes before they are sprayed with the diazotized amines. If this is not done, the residual acid present on the paper interferes with the development of the characteristically colored dyes. It is essential that great care be taken to protect these chromatograms from contamination with vapors of phenol which may be present in many laboratories, particularly if phenolic systems are being used for other chromatographic work. Otherwise, background color develops and conceals the presence of many compounds which may be present in small amounts.

The chromatographic behavior of the authentic compounds is listed in Table I. The most satisfactory combination of systems for two-dimen-

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[1] Differences may be noted in R_F values obtained by workers in other laboratories when conditions reported here are followed exactly. The low relative humidity (20 to 60 per cent) and atmospheric pressure (640 mm. of Hg) at the altitude of this laboratory (5000 feet) undoubtedly should lead to differences in the observed R_F, but the relative values of different compounds should be the same.

sional chromatography has proved to be isopropyl alcohol-aqueous ammonia-water (8:1:1) for the first run, followed by benzene-propionic acid-water (2:2:1, organic phase) as the second system. Satisfactory results are not obtained if the order in which the systems are used is reversed. The location of the known substances on the two-dimensional chromatograms may be estimated from the R_F values listed in Table I. A few substances show slightly altered behavior when chromatographed in the mixture found in urine, but the tentative identification of each has been confirmed by the behavior of authentic material added to urine extracts.

Examination of Phenolic Acids of Urine—An inspection of the chromatographic behavior of the authentic phenolic acids studied here, as revealed by the R_F values of Table I, shows that one-dimensional chromatography is of little use in examining a complex mixture such as that presented by the phenolic acids of urine and that a two-dimensional system is essential. It is necessary to separate and concentrate the acids by extraction into an organic solvent, since chromatography of urine itself gives little indication of their presence; they are present in small amounts, and other compounds interfere with their chromatographic characteristics. In order to compare the excretion of the compounds by different individuals and by the same person at different times, the amount of extract chromatographed is based upon the creatinine content of the urine sample extracted. An assay of the preformed creatinine content of a sample is made, and, when the final organic extract is prepared, it is diluted to correspond to a definite amount of creatinine in the original sample. With samples containing at least 100 mg. of creatinine, an extract can conveniently be prepared in a concentration such that 1 ml. corresponds to 10 mg. of creatinine in the original sample. With smaller samples of urine, such as are obtained from infants, a final concentration of 1 ml. per mg. of creatinine can usually be prepared.

Other investigators routinely have subjected urine samples to acid hydrolysis before extraction (4, 7, 8). Only a small proportion of the organic acids in human urine has proved to be conjugated in forms which do not extract into organic solvents; on the other hand, some of the urinary compounds are sensitive to acid. Unhydrolyzed urine has been used for all of the present work, and care has been taken to avoid the decomposition of compounds which might be unstable to acid conditions. In order to minimize decomposition of unstable compounds, batch extractions have been carried out rather than continuous extractions. For routine purposes, 3×0.5 volume of portions of ethyl acetate[2] have been used to ex-

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[2] (a) The solubility of many of the polar phenolic acids is more favorable in ethyl acetate than in ether, (b) ethyl acetate does not form peroxides which might oxidize sensitive compounds, and (c) for routine use in the laboratory the fire hazard with ether would be considerable.

TABLE I

Chromatographic Behavior of Phenolic Acids

Solvent systems, Ipr-NH₃, isopropyl alcohol-aqueous ammonia-H_2O, 8:1:1; Bz-Prop acid, benzene-propionic acid-H_2O, 2:2:1 (organic phase); aq. KCl, 20 per cent aqueous KCl; Bu-Ac acid, n-butanol-acetic acid-H_2O, 8:2:2; Bu-Pyr-Diox, n-butanol-pyridine-dioxane-H_2O, 70:20:5:5. *Reagents*, DzSA, diazotized sulfanilic acid (10); DzPNA, diazotized p-nitroaniline (4). *Abbreviations*, lt., light; dk., dark; R, red; O, orange; Y, yellow; Bl, blue; P, purple; G, gray; Br, brown; Bk, black.

Acid	R_F					Qualitative color reactions			
	Ipr-NH₃	Bz-Prop acid	Aq. KCl	Bu-Ac acid	Bu-Pyr Diox-	Ultra-violet light	DzSA	DzPNA	Mis-cella-neous
o-Hydroxy-									
benzoic	0.78	0.88	0.66	0.90	0.43	Bl	Lt. Y	Lt. R	
hippuric	0.22	0.43	0.68	0.86	0.30	"	" "	" "	O*
phenyl-									
acetic	0.76	0.57	0.86	0.91	0.49		Dk. O	Dk. P	
mandelic (a)†	0.58	0.13	0.82	0.54	0.30	Dark	Y	Lt. R	
	(0.68)		(0.88)	(0.68)					
phenylpro-									
pionic	0.70	0.74	0.77	0.91	0.72		O	Dk. P	
cinnamic	0.35	0.70	0.44	0.93	0.75	White	Lt. O	" "	
phenyl-									
lactic	0.59	0.29	0.84	0.85	0.28		" "	Lt. "	
phenyl-									
pyruvic (b)†	‡	0.21	0.85	0.70	0.43	White	O	P	G§
phenyl-									
pyruvic									
(lactone)	0.54	0.88	0.44	0.85	0.87		Lt. O	Lt. Br	
m-Hydroxy-									
benzoic	0.39	0.53	0.70	0.88	0.50	Dk. Bl	Dk. Y	Dk. R	
hippuric	0.33	0.09	0.75	0.78	0.19	" "	" "	" "	O*
phenyl-									
acetic	0.46	0.49	0.83	0.92	0.46		Lt. O	Lt. P	
mandelic	0.37	0.07	0.87	0.68	0.19		Dk. Y	Dk. R	
phenylpro-									
pionic	0.54	0.61	0.77	0.92	0.68		Lt. O	Lt. P	
cinnamic	0.44	0.58	0.39	0.93	0.60		Dk. Y	Dk. R	
phenyl-									
lactic	0.51	0.12	0.86	0.82	0.21		Lt. O	Lt. P	
phenyl-									
pyruvic (c)†	‡	0.13	Streak	0.74	0.50‡	Bl	Dk. Y → Br	G	Br§
p-Hydroxy-									
benzoic	0.23	0.55	0.54	0.87	0.68	Dark	Dk. Y	R	
hippuric	0.15	0.07	0.72	0.78	0.18	"	Lt. O	Lt. R	O*
phenyl-									
acetic	0.42	0.49	0.82	0.92	0.50		P	Bl-P	
mandelic	0.33	0.07	0.87	0.68	0.16		Dk. Y	R	

Table I—*Continued*

Acid	R_F					Qualitative color reactions			
	Ipr-NH₂	Bz-Prop acid	Aq. KCl	Bu-Ac acid	Bu-Pyr-Diox	Ultra-violet light	DzSA	DzPNA	Miscellaneous
p-Hydroxy-phenylpro-pionic	0.51	0.61	0.73	0.93	0.69		P	P	
cinnamic	0.2S	0.5S	0.31	0.93	0.67	Dark	R	Bl-G	
phenyl-lactic	0.45	0.13	0.S5	0.S2	0.19		P	P	
phenyl-pyruvic†	‡	0.15	Streak	0.78	‡		O-R	Dk. G	G§
3-MeO-4-OH-benzoic	0.22	0.S0	0.66	0.S9	0.60	White	R-O	" P	
hippuric	0.16	0.1S	0.66	0.74	0.16		Lt. O-R	P	O*
phenyl-acetic	0.39	0.66	0.82	0.S8	Streak	Lt. Bl	" R	G-Br	G§
mandelic	0.2S	0.16	0.84	0.71	0.20		O → R	Bl-G	"
phenylpro-pionic	0.43	0.81	0.74	0.91	0.65		R-P	G	Br§
cinnamic	0.27	0.S0	0.35	0.S8	0.63	Bl	P (fades)	Bl-G	
phenyl-pyruvic	‡	0.25	Streak	0.73	‡	"	O-R	G	G-Br§
p-Hydroxy-cinnamoyl-glycine	0.23	0.09	0.46	0.80	0.20	Dk. Bl	Dk. R	Bl-G	O*
Feruloylgly-cine	0.18	0.19	0.35	0.75	0.18	Bl	P (fades)	"	"
Protocate-chuic	0.06	0.16	0.73	0.83	Streak	Dark	Lt. Br	Br	Bk§
Gentisic	0.68	0.26	0.53	0.89	0.34	Bl	White	Lt. Y	"
α-Resorcylic	0.24	0.09	0.64	0.78	0.39	Dk. Bl	Br-O	Br	
β-Resorcylic	0.39	0.38	0.48	0.92	0.34	Bl	Dk. Br	Dk. Br	
γ-Resorcylic	0.77	0.11	0.50	0.54	0.48	Dark	" "	Lt. "	
Gallic	0.01‡	0.02	Streak	0.60	0.29‡	"	Br	Dk. "	Bk§
Syringic	0.1S	0.79	0.57	0.87	‡		R (fades)	Y	G§
Homoproto-catechuic	0.06‡	0.15	0.75	0.76	Streak	Dark	Lt. Br	Br	Bk§
Homogentisic	0.05‡	0.09	0.90	0.75	0.34		" "	"	"
Caffeic	0.05‡	0.19	0.36	0.78	0.53	Bl	" "	"	"
Kynurenic	0.52	0.16	0.37	0.55	0.21	"			
Xanthurenic	0.0S	0.03	0.24	0.53	0.23	"	Dk. R	P	
3-Hydroxy-anthranilic	0.12‡	0.3S	Streak	0.85	0.65	"	Lt. Br	G	Bk§
5-Hydroxin-doleacetic	0.32	0.16	0.58	0.76	0.45	Faint pink	Dk. R	G	Bl‖ Bk§

TABLE I—*Concluded*

* *p*-Dimethylaminobenzaldehyde in acetic anhydride (11).

† (*a*) *o*-Hydroxymandelic acid gives two yellow spots in many of the solvent systems. One of these perhaps represents the lactone and one the acid. (*b*) *o*-Hydroxyphenylpyruvic acid was used in the form of its lactone, 3-hydroxycoumarin. Experiments in which the lactone was allowed to stand in the presence of excess 0.1 N NaOH and then neutralized before applying to chromatograms showed the presence of other components. One of these, in some systems, showed properties expected for the free pyruvic acid and the R_F values for it are listed. (*c*) The pyruvic acids bore evidence of decomposition and the presence of several components in many systems. This behavior perhaps occurs as a result of the keto-enol tautomerism of these compounds and, in addition, of their reactivity. Special conditions will be necessary for the chromatography of these compounds.

‡ Decomposes.

§ Ammoniacal AgNO₃ (10).

‖ *p*-Dimethylaminobenzaldehyde in aqueous HCl (10).

tract urine which was acidified to pH 1 to 2 and saturated with sodium chloride. Reextraction of samples that had been subjected to this treatment showed that small amounts of the more polar compounds are not completely extracted. This standard procedure has been used routinely for the examination of normal and of pathological urines, however, and marked variations in the amounts of polar metabolites present in different samples are evident, even though the extraction of some of the compounds is not quantitative.

Procedure by Which Phenolic Acids of Urine Are Prepared for Chromatography—The volume of a sample of urine is measured and the preformed creatinine is estimated with the Jaffe procedure. An aliquot corresponding to a definite amount of creatinine is diluted so that it contains 0.5 to 0.8 mg. of creatinine per ml. The diluted urine is chilled in an ice bath, acidified to pH 1 to 2 (indicator paper) by the careful addition of concentrated HCl, and saturated with NaCl (approximately 26 gm. per 100 ml.). It is then extracted with three successive 0.5 volume portions of ethyl acetate. The first extraction is frequently made difficult by the formation of an emulsion; this may be broken by separating as much as possible of the clear portions of the aqueous and organic layers and centrifuging the residual emulsion. The second and third extractions with ethyl acetate usually proceed smoothly. The combined ethyl acetate solutions are extracted by *prolonged* and thorough shaking with small portions of 10 per cent NaHCO₃ solution. An initial extraction with an amount such that 1 ml. corresponds to about 20 mg. of creatinine in the original sample of urine, followed by two more extractions with half that volume for each extraction, usually proves satisfactory. The combined bicarbonate extracts are chilled in an ice bath, acidified to pH 1 to 2 by the addition of concentrated HCl, saturated with salt, and then extracted with successive

small portions of ethyl acetate. The ethyl acetate extracts may be collected in a graduated cylinder of an appropriate size, and the final volume of ethyl acetate may then be adjusted to correspond to a definite amount

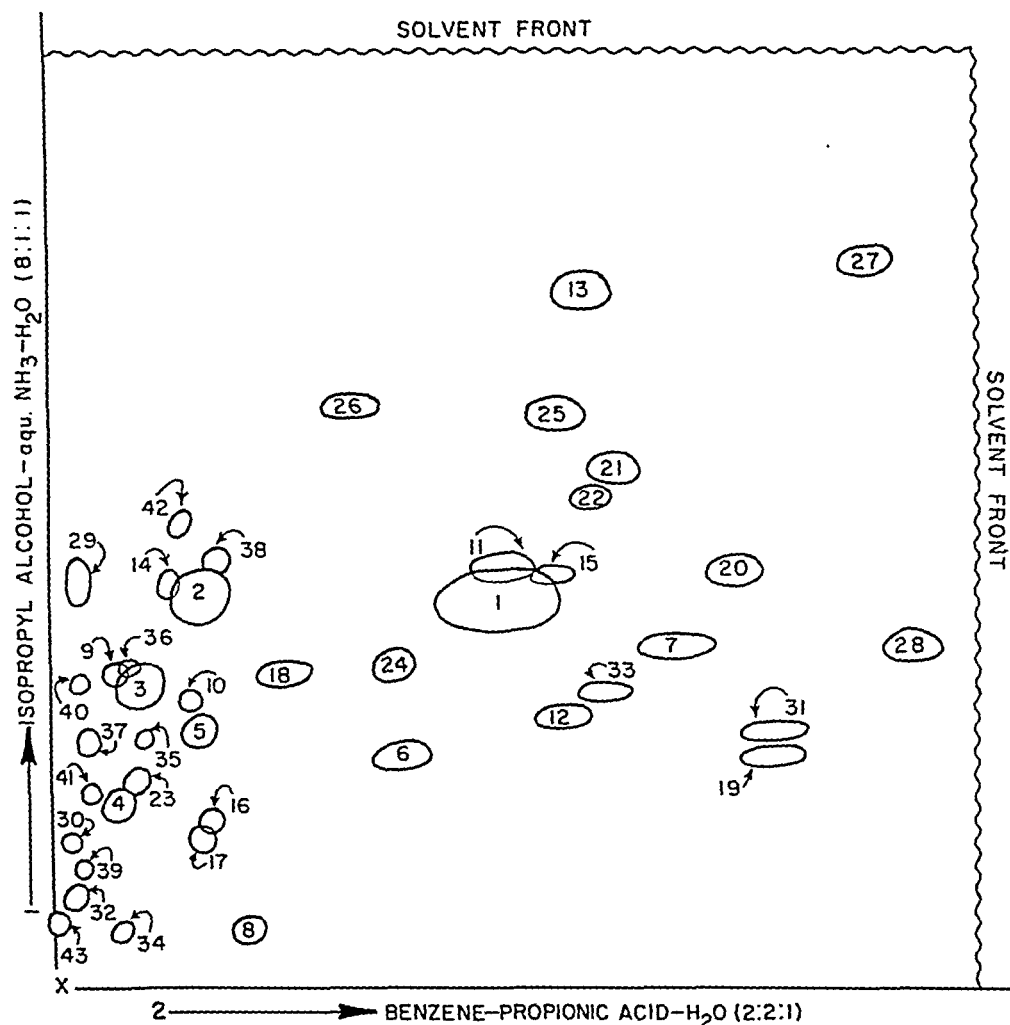FIG. 1. Phenolic acids in human urine. The numbers refer to the compounds listed in Table II.

of creatinine in the original urine. When it is desired to store extracts, they may be dried by adding anhydrous Na_2SO_4 and decanting the ethyl acetate into a container; the extract may then be stored in a deep freeze at $-15°$.[3]

[3] Used penicillin vials are excellent containers for storing extracts.

TABLE II

Phenolic Acids in Human Urine

Compound No.	Description	R_F	
		Ipr-NH₃	Bz-Prop acid
1	*p*-Hydroxyphenylacetic acid	0.42	0.49
2	Dark yellow (DzSA); light purple (DzPNA)	0.43	0.16
3*	*m*-Hydroxyhippuric acid	0.33	0.09
4*	*p*-Hydroxyhippuric acid	0.20 (0.15)†	0.07
5	5-Hydroxyindoleacetic acid	0.28	0.16
6*	Salicyluric acid	0.25	0.38
7*	Homovanillic acid	0.37	0.68
8	Dark red (DzSA); gray (DzPNA)	0.06	0.21
9*	*p*-Hydroxymandelic acid	0.34	0.07
10	Orange → red (DzSA); blue (DzPNA)	0.31	0.14
11	*m*-Hydroxyphenylacetic acid	0.45	0.49
12	*p*-Hydroxybenzoic acid	0.29 (0.23)†	0.55
13	*o*-Hydroxyphenylacetic acid	0.75	0.58
14	*p*-Hydroxyphenyllactic "	0.44	0.13
15	*m*-Hydroxybenzoic acid	0.45 (0.39)†	0.47
16*	Feruloylglycine	0.18	0.17
17*	Vanilloylglycine	0.16	0.16
18	Orange-red (DzSA); blue-gray (DzPNA)	0.34	0.25
19	Vanillic acid	0.25	0.78
20	Dihydroferulic acid	0.45	0.74
21*	*m*-Hydroxyphenylpropionic acid	0.56	0.61
22	*p*-Hydroxyphenylpropionic acid	0.53	0.59
23*	α-Resorcylic acid	0.22	0.09
24	Dark yellow (DzSA); red (DzPNA)	0.35	0.37
25	" " " reddish purple (DzPNA)	0.62	0.55
26	Reddish orange (DzSA); light purple (DzPNA)	0.63	0.33
27	Salicylic acid	0.78	0.88
28	Red (DzSA); blue (DzPNA)	0.37	0.93
29	Orange → gray (DzSA); light brown (DzPNA) (pregnancy urine)	0.44	0.03
30	Dark brown (DzSA); purple-brown (DzPNA)	0.16	0.02
31	Ferulic acid	0.29	0.78
32	Dark yellow (DzSA); light brown (DzPNA)	0.10	0.02
33	*p*-Hydroxicinnamic acid	0.33	0.60
34	Purple (DzSA); blue-purple (DzPNA)	0.06	0.07
35*	*p*-Hydroxycinnamoylglycine	0.27	0.10
36	Orange (DzSA); yellow (DzPNA)	0.34	0.07
37	Reddish purple (fades) (DzSA); gray (DzPNA)	0.27	0.04
38	Purple (DzSA); blue-purple (DzPNA)	0.46	0.18
39	Maroon (DzSA); purple (DzPNA)	0.13	0.03
40	Gray (DzSA); blue-gray (DzPNA)	0.33	0.03
41	Light orange-red (DzSA); purple (DzPNA)	0.21	0.04
42	Purple (DzSA); purple (DzPNA)	0.50	0.14
43	Brown (DzSA); gray (DzPNA)	0.07	0.00

Table II—Concluded

* Compounds not previously reported to be normal constituents of urine.

† Compounds in which R_F in urine extracts differs from R_F when measured alone. The values in parentheses are those obtained with the pure compound alone.

For chromatography, an amount of the ethyl acetate solution corresponding to 1 mg. of creatinine in the original urine has usually proved satisfactory. Larger quantities may be chromatographed when samples from individuals who have been eating synthetic diets are prepared, but urine from normal persons on a natural diet usually contains so much hippuric acid that the chromatography of other compounds is disturbed. With urine from patients with phenylketonuria, the presence of large amounts of phenylpyruvic and phenyllactic acids and phenylacetylglutamine makes it impractical to use a sample larger than one corresponding to 0.5 mg. of creatinine. The extracts are applied to paper from a micro pipette with a stream of air at room temperature directed at the paper to aid the evaporation of the solvent. The diameter of the spot of the acids deposited for chromatography should not exceed 7 mm. An overnight run (16 hours) at 25° is required for a 25 cm. solvent advance with the isopropyl alcohol-ammonia system and a 3 hour run with the benzene-propionic acid system.

Fig. 1 is a schematic diagram representing the location of the well defined phenolic acids which are found in most samples of normal human urine. Table II provides a list of these compounds. When the location on the chromatogram and the characteristic reactions correspond to those of known compounds, the material is given a tentative identification. Materials which do not correspond to any of the standard compounds are described by their characteristic color with the diazotized amines. The compounds have been arbitrarily designated by number. When an amount of extract corresponding to 1 mg. of creatinine is chromatographed, Compounds 1 to 10 may be detected in almost all samples of urine in amounts decreasing with increased number. Compounds 11 to 17 may be detected in a majority of the samples, and the remainder of the compounds described is present in only an occasional sample of urine or is present in much smaller amounts.[4]

It should be noted that the alkaline medium used for the first solvent in the chromatography reported here leads to the decomposition of many easily oxidized dihydroxyphenyl compounds. Further work with other

[4] The amounts of the acids excreted daily varied greatly under different circumstances. As a rough approximation, it may be estimated that for most individuals under average conditions the amounts of Compounds 1 to 10 excreted daily range from 20 to 25 mg. down to about 2 mg., and of Compounds 11 to 17 from 5 mg. to less than 0.5 mg.

systems will be necessary to characterize these compounds, among which would be homogentisic, homoprotocatechuic, and caffeic acids, as well as *o*- and *p*-hydroxy- and 3-methoxy-4-hydroxyphenylpyruvic acids.

Two-dimensional paper chromatography of the phenolic acids of human urine reveals a complex mixture of closely related compounds. Approximately 400 normal and pathological samples have been examined; the compounds definitely characterized in this paper are those which have been found to occur in all or at least several samples. Many more phenolic acids have been observed in the course of this work, but are not reported, either because they were observed in only a single sample of urine or because they gave ill defined spots on the chromatograms. In addition, some substances are not reported which have been observed in small amounts and which are apparent only transiently on the freshly sprayed chromatograms.

The preliminary and tentative identification made when the qualitative reactions and chromatographic behavior of a compound in urine extracts correspond to authentic compounds is probably correct. It should be emphasized, however, that final identification must be based upon actual isolation and comparison with the authentic substance. A great many of these compounds are closely related chemically and have similar solubility behavior and qualitative reactions.

The variety of phenolic acids observed in these experiments is of interest because of their number and their nature. Of the twenty-three acids accorded a tentative identification, ten have not been previously reported to occur as normal constituents of urine. Two series of compounds, those containing an *m*-hydroxyphenyl group (Compounds 2, 3, 11, 15, 21) (13) and the 3-methoxy-4-hydroxyphenyl group (Compounds 7, 16, 17, 19, 20, 31) are of particular interest, since a wide-spread metabolic occurrence of them had not been previously suspected.

When the conclusive identification of each compound has been made, it will be important to establish whether it is an end-product of endogenous metabolism, or whether it occurs as a result of the action of intestinal microorganisms or is derived from food constituents. When these factors have been established, it will be possible to examine alterations in the excretion of these phenolic acids that have been observed in phenylketonuria and in some other pathological conditions in an attempt to gain a better understanding of the relation of the abnormal urinary compounds to the pathological state.

SUMMARY

The chromatographic behavior of forty-nine simple phenolic acids is described. The chromatographic properties of forty-three distinctive

phenolic acids that have been observed in samples of urine from 400 individuals, both healthy and ill, are described, and a preliminary identification is given for twenty-three of them.

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BIOLOGICAL SYNTHESIS OF LANOSTEROL AND
AGNOSTEROL*

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There is considerable experimental evidence in support of the view that, in the biogenesis of cholesterol, squalene is an intermediate (1, 2). It has been suggested (3) that the cyclization of the aliphatic triterpenoid hydrocarbon to the tetracyclic ring system of the steroids occurs in a manner which would also rationalize the structure of lanosterol, a naturally occurring 4,4',14-trimethylcholestane derivative (4). In view of its structural relationship to squalene on the one hand and to cholesterol on the other, it has become of interest to investigate the possible rôle of lanosterol, or of related structures having 30 carbon atoms, as intermediates in the biosynthesis of cholesterol. That the synthesis of lanosterol from acetate takes place in mammalian tissues has now been demonstrated. Moreover, as will be shown in the accompanying paper (5), lanosterol serves as a precursor of cholesterol.

At the start of this work, since pure lanosterol was not available to us, tentative information on the biogenesis of C_{30} sterols was obtained by the use of natural "isocholesterol" as carrier. The "isocholesterol" of wool fat, the most abundant source of lanosterol, is known (6) to consist of the four components (Fig. 1), lanosterol, dihydrolanosterol, agnosterol, and dihydroagnosterol, in variable proportions, with lanosterol as the major constituent. From this mixture pure lanosterol can be isolated only by very tedious procedures (6, 7).

For the demonstration of lanosterol biosynthesis, rat liver homogenates prepared according to the method of Bucher (8) were incubated with sodium acetate-1-C^{14} and the unsaponifiable fractions combined with small amounts of "isocholesterol" as carrier. Careful chromatography of the resulting mixture demonstrated that a small but significant percentage of the total C^{14} in the unsaponifiable material remained associated with the "isocholesterol" fraction. When rat liver homogenates were incubated under similar conditions but with prior addition of suspensions of "isocholesterol," the incorporation of acetate carbon into this fraction was mark-

edly enhanced.   This procedure was therefore adopted for the preparation of labeled material in quantities sufficient for further chemical and biological experiments.

Subsequently, lanosterol ($\Delta^{8,24}$-lanostadienol) was isolated from a yeast concentrate and the C^{14}-"isocholesterol" diluted with this pure sterol.   By suitable chemical reactions it was shown that lanosterol was in fact the major radioactive component of the biosynthetic product.   Evidence for the biosynthesis of agnosterol in the same liver system was also obtained. Hitherto the presence of lanosterol in liver tissue has not been observed. The experiments reported here clearly indicate that at least two C_{30} sterols, lanosterol and agnosterol, are natural constituents of liver, though in very small amounts.
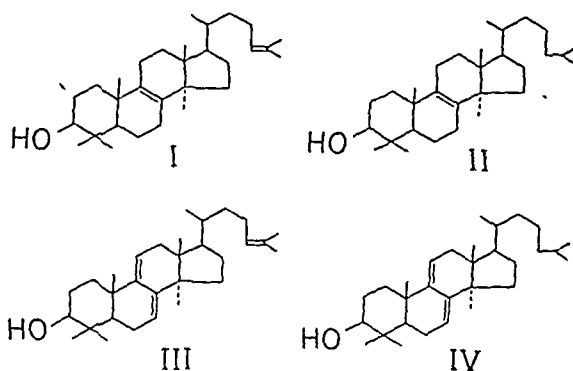
FIG. 1. Constituents of wool fat sterol mixture (isocholesterol).   I, lanosterol; II, dihydrolanosterol; III, agnosterol; IV, dihydroagnosterol.

EXPERIMENTAL

Materials

"Isocholesterol" was supplied generously by Eli Lilly and Company. Lanosterol (m.p. 140–141°) was isolated by chromatography from a concentrate of the unsaponifiable fraction of yeast from which most of the ergosterol had been removed previously by crystallization (9).   The yeast concentrate was kindly provided by Professor L. Ruzicka of Zurich.   Generous samples of agnosterol and dihydroagnosterol were made available to us by Professor D. H. R. Barton, Birkbeck College, London.   The diphosphopyridine nucleotide (DPN) was a product of the Pabst Brewing Company, 95 per cent purity.   The alumina was Merck, chromatographic grade of activity II.   All solvents used in the chromatographic procedures were anhydrous.

Rat Liver Homogenates—Female rats weighing approximately 100 gm. were killed by a blow on the head and the livers homogenized according to the procedure of Bucher (8) in a 0.08 M phosphate buffer (pH 7.4) con-

taining nicotinamide (0.03 M) and magnesium chloride (0.0048 M). The supernatant fluid obtained after homogenization and centrifugation at low speed (700 × g) was divided into 2 ml. aliquots. In control experiments 0.2 ml. of 0.01 M DPN and 0.2 ml. of 0.1 M sodium acetate-1-C^{14} were added to each aliquot. For experiments involving additions of carrier sterols to the homogenates prior to incubation, suspensions of the steroids were prepared in the following manner. The sterol (10 mg.) was ground to a thin paste with the minimum of Bucher phosphate buffer containing 0.5 per cent bovine serum albumin. The paste was then diluted to 2.0 ml. with the same 0.5 per cent serum albumin buffer solution and transferred to a Raytheon magnetostriction oscillator in which it was subjected to an oscillation frequency of 9 kc. for 30 to 90 minutes, depending upon the time required to reduce the bulk of the solid to a satisfactory emulsion. Suspensions prepared in this way were generally found to remain stable at 0° for 24 hours and could be stored in the frozen state indefinitely. The concentration of sterols could be assumed to be approximately 5 mg. per ml. 0.1 ml. of this suspension was added to each 2 ml. of homogenate. When the homogenates were incubated with 3 times the amount of sterol suspension, essentially the same results were obtained.

Incubations were carried out in a Dubnoff shaker in an atmosphere of oxygen at 37° for 5 hours. After incubation the homogenates were saponified with 30 per cent potassium hydroxide at 25° for 12 hours and the unsaponifiable fractions extracted with petroleum ether. Total C^{14} incorporation was measured by plating aliquots as infinitely thin samples and counting in a gas flow counter.

Incorporation of Acetate into Lanosterol in Control Experiments—The following is one of several preliminary experiments which showed that the unsaponifiable fraction contained in small percentage a labeled substance which was chromatographically inseparable from "isocholesterol." 6 ml. of homogenate prepared as described above, and incubated with sodium acetate-1-C^{14} (0.025 mc. per mmole), yielded 1.8 mg. of unsaponifiable material containing a total of 4700 c.p.m. 10 mg. of "isocholesterol," m.p. 137–140°, and 10 mg. of pure cholesterol were added and the mixture rechromatographed on alumina to give the fractions in Table I.

Fractions 6 and 7, 7.5 mg., corresponded approximately in both weight and melting point with the added carrier "isocholesterol." They were therefore presumed to consist mainly of this material. For the purpose of washing out, these fractions were combined with 10 mg. of pure cholesterol and again chromatographed on 3 gm. of alumina (Table II). Fractions 4 to 6, 6.1 mg., contained a total of 46 c.p.m. or 7.5 c.p.m. per mg. After recrystallization from methanol, these combined fractions gave 4 mg. of material, m.p. 135–140°, with a specific activity of 6.5 c.p.m. per mg.

The cholesterol fractions of the second chromatogram (Fractions 8 to 11, Table II) contained no detectable radioactivity.

Incubations with Carrier "Isocholesterol"—40 ml. of rat liver homogenate prepared as described were divided into twenty aliquots of 2 ml., and 0.2 ml. of 0.1 M sodium acetate-1-C^{14} (1 mc. per mmole) was added to each. Two flasks were taken as controls (Experiment B), and to each of the re-

TABLE I

Alumina Chromatography of Unsaponifiable Fraction from Liver Homogenates after Incubation with Acetate-1-C^{14}

Fraction No.	Solvent	Volume	Weight of fraction	Total activity	Appearance; m.p.
		ml.	*mg.*	*c.p.m.*	°C.
1	Petroleum ether, b.p. 60–68°	30	0.2	25	Oil
2	Benzene-petroleum ether 1:4	10			
3	Benzene-petroleum ether 2:3	10	0.1	25	Gum
4	Benzene	10	0.1	13	"
5	Ether-benzene 1:50	30	0.8	49	Semisolid
6	" 1:20	30	3.5	39	White solid 134–139
7	" 1:20	30	4.0	57	" " 135–138
8	" 1:20	10	0.9	40	" " 125–135
9	" 1:9	10	1.2	109	Semisolid 115–130
10	" 1:9	10	1.2	277	White solid 130–135
11	" 1:9	10	1.5	377	" " 135–145
12	" 1:9	10	2.0	361	" " 140–148
13	" 1:9	30	4.0	830	" " 135–145
14	" 1:9	10	1.6	173	" "
15	" 1:4	20	0.8	186	" " 130–142
16	Ether	20	0.3	74	
17	Methanol-ether 2:3	20	0.2	190	White semisolid

maining eighteen flasks (Experiment A) was added 0.1 ml. of a suspension of "isocholesterol" (5 mg. per ml.). After incubation, the homogenates were saponified and the unsaponifiable fractions extracted with petroleum ether, yielding a total of 3 mg. from the combined controls and 27 mg. from the homogenates treated with "isocholesterol." The total activities estimated from infinitely thin aliquots were 154,000 and 1,250,000 c.p.m., respectively.

The combined unsaponifiable fractions of the controls were mixed with 5 mg. of cholesterol and 5 mg. of lanosterol; those of the "isocholesterol"-treated preparations were not further diluted. The two batches of un-

saponifiable material were now chromatographed in parallel on columns of 10 gm. of alumina, the fractions being identified according to their appearance and levels of activity, as seen in Table III.   For purposes of direct comparison of Experiments A and B, the total C^{14} activities for Experiment A in Table III were divided by 9 because the scale of Experiment A was 9 times that of the control Experiment B.   Fractions 6 to 13 from Experiment A combined weighed 5.1 mg. and had a specific activity of 24,000

TABLE II

Chromatographic Separation of "Isocholesterol" and Cholesterol

The volume for Fractions 1 to 12 was 10 ml.

Fraction No.	Solvent		Weight of fraction	Total activity	Appearance; m.p.
			mg.	*c.p.m.*	*°C.*
1	Benzene				
2	Ether-benzene 1:20				
3	"	1:20	0.7	6	White solid
4	"	1:20	2.2	13	" " 133–139
5	"	1:20	2.3	18	White solid 133–139
6	"	1:20	1.6	15	White solid 134–139
7	"	1:20	0.9	6	White solid
8	"	1:20	1.1	0.5	" " 135–147
9	"	1:20	2.4	0	White solid 140–148
10	"	1:20	3.7	0	White solid 145–148
11	"	1:4	2.6	0	White solid 144–148
12	"	1:4	0.2	0	

c.p.m. per mg.   After dilution with 5 mg. of pure lanosterol[1] and crystallization from methanol, 8.2 mg. of needles were obtained which were further diluted to 82 mg. by addition of pure lanosterol.   One recrystallization now gave 73.5 mg. of needles, m.p. 140–141°, having 1300 c.p.m. per mg. From the proportion of carrier lanosterol added, assuming no loss of ac-

[1] The material termed "pure lanosterol" was shown to absorb 1 mole of hydrogen and therefore consisted entirely of material with the unsaturated side chain.   Careful ultraviolet absorption measurements revealed the presence of a maximum of 1.5 per cent of substances such as agnosterol, having the $\Delta^{7 \cdot 9}$ structures; otherwise the physical properties of the material were those recorded in the literature for pure lanosterol.

tivity on crystallization, the calculated specific activity of this material is 1200 c.p.m. per mg.

To obtain sufficient lanosterol for chemical characterization, this labeled

TABLE III

Chromatogram of Unsaponifiable Fraction from Liver Homogenates after Incubation with and without Carrier "Isocholesterol"

Fraction No.	Solvent		Volume	Experiment A "Isocholesterol"-treated homogenate		Experiment B Control	
				Total activity	Appearance	Total activity	Appearance
			ml.	*c.p.m.*		*c.p.m.*	
1	Hexane		30	1370	Oil	128	Oil
2	Benzene		30	1550	Gum	684	Gum
3	Ether-benzene 1:99		30	200		160	
4	"	1:99	30	650		370	
5	"	1:99	30	330		260	
6	"	1:99	10	740	Trace solid	48	Trace solid
7	"	1:99	10	2500	White "	100	White "
8	"	1:99	10	2340	" "	78	" "
9	"	1:99	10	2660	" "	136	" "
10	"	1:99	10	2440	" "	126	" "
11	"	1:99	10	1660	" "	136	" "
12	"	1:99	10	500	Trace "	104	Trace "
13	"	1:99	10	570	" "	156	" gum
14	"	1:99	20	160	" gum	370	" "
15	"	1:99	30	500		490	
16	"	3:97	20	180	Trace gum	110	Trace gum
17	"	3:97	30	700	" solid	1,900	" solid
18	"	1:9	20	2500	White "	2,100	White "
19	"	1:9	20	9000	" "	7,800	" "
20	"	1:9	20	6000	" "	8,000	" "
21	"	1:4	20	8500	" "	21,000	" "
22	"	1:4	20	7200	" "	15,400	" "
23	"	1:4	20	3200	" "	14,500	" "
24	"	1:4	20	2600	" "	3,400	" "
25	Ether		20	1640	" "	2,100	" "
26	"		20	1330	" "		
27	"		40	2600	Trace "	636	Trace solid
28	Ether-methanol 1:1		40	4500	" "	12,000	" "

material was subjected to further dilutions and recrystallizations: (1) 50 mg. of lanosterol (1300 c.p.m. per mg.) were diluted to 500 mg. with pure lanosterol. Crystallization from acetone-methanol gave 460.3 mg. of lanosterol, the specific activity of which, by combustion and counting as infinitely thick samples of barium carbonate, was 97 c.p.m. (2) The foregoing material was diluted with its own weight of pure lanosterol and again

recrystallized to yield 776 mg. of lanosterol, m.p. 140–141°. The specific activity as infinitely thick samples of barium carbonate was now 48.8 c.p.m. The chemical work reported below was carried out with this product.

Chemical Characterization of Labeled Lanosterol—The specific activities of the lanosterol derivatives described in this section are expressed as counts per minute of infinitely thick samples of barium carbonate.

Dehydration and Rearrangement of Ring A—Lanosterol (48.8 c.p.m.) was hydrogenated under neutral conditions in ethyl acetate solution in the presence of a platinum catalyst to give dihydrolanosterol, m.p. 143–146° (10). Treatment of this product with phosphorus pentachloride in anhydrous petroleum ether, followed by chromatography and recrystallization from acetone, gave the known isopropylidene rearrangement product (V, Fig. 2), m.p. 140–143°, $[\alpha]_D$ +65°; specific activity, 51 c.p.m. Previously reported (10), m.p. 143–144°, $[\alpha]_D$ +67°.

3β-Acetoxylanost-8-ene-7,11-dione[2]—Dihydrolanosteryl acetate (48.8 c.p.m.) was oxidized with chromic acid to give 3β-acetoxylanost-8-ene-7,11-dione (VII, Fig. 2), m.p. 156–158°, previously reported (6), m.p. 156.5–158.8°; specific activity, 50 c.p.m.

Degradation of Side Chain of Lanosterol—Lanosterol (48.8 c.p.m.) was converted to the 3β-24,25-triol (II, Fig. 2) by treatment with osmium tetroxide, according to the method of Wieland and Benend (11). The triol was obtained as plates, m.p. 167–177°, $[\alpha]_D$ +45°; specific activity, 45 c.p.m. Oxidation of the triol was carried out by a modification of the method described by the above authors. The foregoing triol (250 mg.) was dissolved in the minimum of glacial acetic acid, and 375 mg. of lead tetraacetate (1.5 moles per mole of sterol), also dissolved in the minimum of acetic acid, were added. Nitrogen was passed through the solution for 20 hours at 25° and the issuing gas passed through two traps connected in series and cooled in liquid nitrogen. The traps were periodically detached and their contents washed out with 50 ml. quantities of Van Slyke mercuric sulfate reagent (12) for the precipitation of acetone. These solutions were refluxed for 1 hour to precipitate the acetone-mercuric sulfate complex. From the combined precipitates (300 mg., 60 per cent of the theory), acetone was regenerated by boiling with 10 per cent hydrochloric acid and reprecipitated as the mercuric sulfate complex (170 mg.). This material was subjected to wet combustion by the method of Van Slyke and Folch (13), and the carbon dioxide precipitated as barium carbonate; specific activity, 34 c.p.m. Assuming by analogy with the data on acetate utilization for cholesterol (14) that only the carbonyl carbon of the acetone contains C^{14} and that lanosterol is derived from 18 methyl and 12 carboxyl

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[2] Experiments carried out by Dr. T. Lyssy in this laboratory.

carbon atoms of acetic acid, the theory requires $48.8 \times 30 / 12 \times 3 = 41$ c.p.m.

3β-Acetoxylanost-8-en-24-al—2.5 hours after the beginning of the above treatment with lead tetraacetate, a crystalline precipitate of 3β-acetoxy-lanost-8-en-24-al (III, Fig. 2) was separated from the solution by filtration, and the filtrate returned to the reaction vessel in which the removal of acetone in a nitrogen stream was continued. The aldehyde (100 mg.) melted with decomposition over the range, 155–185°. Recrystallization from chloroform and acetic acid gave prisms, m.p. 210–220° (decomposition), $[\alpha]_D$ +57°. It readily gave a yellow 2,4-dinitrophenylhydrazone and
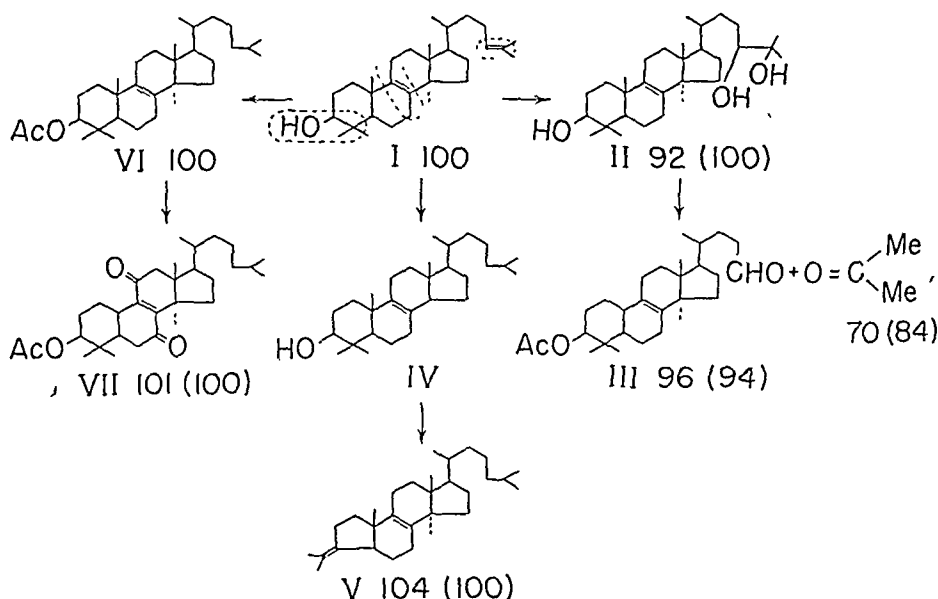

FIG. 2. Isotope concentrations in lanosterol and derivatives. The calculated values are given in parentheses.

showed absorption bands in the infra-red at 2540 cm.$^{-1}$ (C—H stretching of aldehyde), a strong band at 1720 cm.$^{-1}$ (C=O of both the aldehyde and acetoxy groups), and a broad band at 1250 cm.$^{-1}$ (ester —C—O—). No hydroxyl band was present. Found, C 78.78, H 10.79; $C_{29}H_{46}O_3$ requires C 78.6, H 10.47. The specific activity of the compound as an infinitely thick sample of $BaCO_3$ was 47 c.p.m.

The C^{14} analyses of the various degradation products of lanosterol are summarized in Fig. 2. Values of the specific activities of the derivatives are recalculated and compared with an assigned value of 100 for that of the lanosterol.

Incubation with Various Components of "Isocholesterol"—Incubation and chromatographic procedures were the same as those described above. The levels of acetate incorporation into the lanosterol fraction of controls and

of homogenates incubated with either natural or reconstituted "isocholesterol" were compared with those obtained on treatment of the homogenates with suspensions of the various individual components of wool fat sterol. Suspensions of synthetic "isocholesterol" were prepared by mixing 45 parts of lanosterol, 45 parts of dihydrolanosterol, 5 parts of agnosterol, and 5 parts of dihydroagnosterol. In all the experiments given in Fig. 3, the total quantity of sterol added to the incubation mixtures was 1.5 mg. per 2 ml. of homogenate.

Isolation of Agnosteryl Acetate from Labeled "Isocholesteryl" Acetate— "Isocholesterol" obtained by incubation of a homogenate with carrier

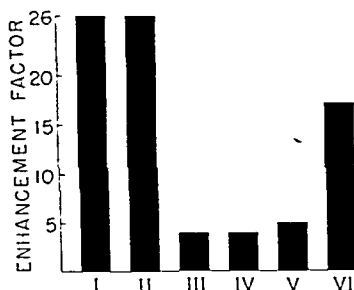
Fig. 3   Fig. 4

FIG. 3. Enhancement of acetate incorporation into "isocholesterol" in liver homogenates in the presence of carrier sterols. The heights of the bars represent the ratio of total counts per minute in the "isocholesterol" fraction obtained from incubations with carrier sterol, to those obtained in control experiments. I, natural "isocholesterol;" II, reconstituted "isocholesterol;" III, lanosterol; IV, dihydrolanosterol; V, agnosterol; VI, lanosterol, 4 parts, plus agnosterol, 1 part.
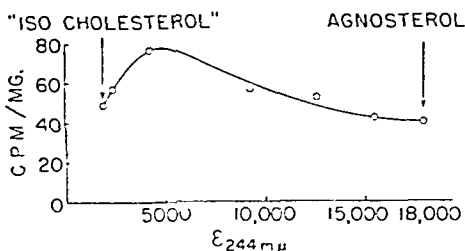
FIG. 4. Isolation of agnosteryl acetate by crystallization of "isocholesteryl" acetate.

"isocholesterol" as described above was diluted and acetylated to yield 7.5 gm. of "isocholesteryl" acetate, having 50 c.p.m. per mg. as an infinitely thin sample. Crystallization of this material from ethyl acetate, according to Windaus and Tschesche (7), results in an enrichment of agnosterol in the less soluble fraction. In the present experiments pure agnosteryl acetate could be obtained after six crystallizations, provided that crystallization was allowed to take place slowly. At each stage the content of agnosteryl acetate was determined by measuring the light absorption at 244 mμ, and the radioactivity determined by counting aliquots as infinitely thin samples. The final product was pure agnosteryl acetate, m.p. 175–179°; ϵ_{244} = mμ 18,000 (15); specific activity as infinitely thin aliquots, 40 c.p.m. per mg. The variation of specific activity with intensity of light absorption at 244 mμ is given in Fig. 4.

DISCUSSION

When liver homogenates are incubated with labeled acetate, about two-thirds of the C^{14} in the unsaponifiable fraction is ordinarily recovered as cholesterol. Attempts to account for the remainder of the radioactivity in the form of known steroids have hitherto been largely unsuccessful. In regard to sterols which are known to accompany cholesterol in animal tissues (cholestanol (16), Δ^7-cholestenol (17), 7-dehydrocholesterol (18), 3β-5α-dihydroxy-6-ketocholestanol (19), and 3β-5α-6β-trihydroxycholestane (20)), no evidence exists that they are in any large measure responsible for the remainder of the activity. In the course of the present work, the unsaponifiable fractions after incubation of the homogenates with labeled acetate were carefully chromatographed and it was invariably found that the major part of the radioactivity was associated with four well defined fractions. Two principal fractions, less polar than cholesterol, could be eluted in turn with petroleum ether and benzene. By analogy with *in vivo* experiments (1) and those employing the perfused liver (21), part of the activity in the petroleum ether eluate may be expected to reside in squalene. The benzene fraction remains completely unidentified. The radioactivity associated with this fraction was found to travel chromatographically with ambrein, but chemical evidence indicates that the two materials are not identical. After elution of cholesterol, another fraction of high specific activity emerges from the column with ether-methanol mixtures.

After it had been ascertained that the components of "isocholesterol," though inseparable from each other, could be chromatographically separated from cholesterol, it became possible to show the presence of a fifth fraction, slightly less polar than cholesterol. This "lanosterol" fraction under the conditions of the control experiments was very small, both in weight and in C^{14} content, and in fact its presence could be demonstrated only by chromatography of the unsaponifiable material with lanosterol or "isocholesterol" added as carrier.

The data of Tables I and III demonstrate that in the control exper ments not more than 1 per cent of the total C^{14} content of the unsaponifiab. fraction could be attributed to lanosterol or other components of "isc cholesterol." It was, however, clearly shown by a chromatographic wasl ing out procedure (Table II) that the radioactivity of the lanosterol fractio was not due to contamination by cholesterol.

On the assumption that lanosterol might be an intermediate with a hig rate of turnover, it was considered likely that newly synthesized lanosterc could be trapped and the yields of C^{14} increased by the presence of carrie during the incubation. Data such as those in Table III, Experiment A illustrate that a considerable enhancement of the incorporation of C^{14} int the lanosterol fraction does indeed result from the addition of carrier "iso

cholesterol" to the homogenate prior to incubation. In the particular experiment cited, the C^{14} content of the lanosterol fraction accounted for 10 per cent of the total in the unsaponifiable fraction, corresponding to a 30-fold increase compared with the control.

Since the "isocholesterol" used as a carrier consisted of four C_{30} sterols which were inseparable by our chromatographic procedure, it was clearly possible for any or all of the components to contain C^{14}. In the expectation that one of the radioactive sterols was lanosterol, the labeled "isocholesterol" (Fractions 6 to 13, Table III) was diluted with pure lanosterol and recrystallized several times without change of specific activity. This material was converted to derivatives which were chosen to demonstrate, as far as possible, that the radioactivity was associated with a compound which had the typical structural features of lanosterol. These derivatives were (1) the C_{27} aldehyde produced together with acetone by oxidative degradation of the side chain, (2) the 7,11-diketone formed on oxidation of dihydrolanosteryl acetate, and (3) the isopropylidene rearrangement product obtained on dehydration of dihydrolanosterol with PCl_5. Through all of these transformations the radioactivity of the starting material was retained (Fig. 2).

The specific activities of the C_{27} aldehyde and acetone (III, Fig. 2) indicated that all the radioactivity of the starting material resided in substances unsaturated in the 24:25 position. If compounds having a saturated side chain such as dihydrolanosterol or dihydroagnosterol had contributed to any appreciable extent to the radioactivity of the starting material, the C^{14} content of these degradation products would have been proportionately reduced.[3] The concomitant dehydration and rearrangement of ring A to give isopropylidene derivatives of the type V (Fig. 2) are characteristic of the 3β-hydroxy-4,4'-dimethyl sterols and triterpenes. From the formation of this well characterized derivative without alteration of specific activity, it can therefore be concluded that this moiety also is present in the radioactive starting material. The 7,11-diketo-Δ^8 compound (VII, Fig. 2) is an expected product of the chromic acid oxidation of the Δ^8-C_{30} sterols, but is also formed from the corresponding $\Delta^{7,9}$-dienes under similar conditions (6). The unaltered radioactivity in this derivative therefore does not distinguish between lanosterol and agnosterol as the radioactive substance. The relative isotope concentrations of these two components will be discussed below.

[3] In a preliminary experiment the C_{30} sterols with saturated side chains were chemically separated from the labeled "isocholesterol" and shown to account for a maximum of 5 per cent of the radioactivity in the mixture. Since the content of saturated sterols in "isocholesterol" is 40 per cent, their specific activity is calculated to be at most 12.6 per cent of that of the total mixture.

In experiments involving carrier dilution, contamination by substances of closely similar structure cannot be rigidly excluded. However, the close correspondence between the experimental and calculated values for the specific activities of all the derivatives makes it highly improbable that appreciable radioactivity resides in compounds other than lanosterol or agnosterol.

Evidence that, under the conditions of our experiments, both the lanosterol and the agnosterol became labeled is presented in Fig. 4. Crystallization of a large batch of labeled "isocholesteryl" acetate (ϵ_{244} = 2000; specific activity, 50 c.p.m. per mg.) yielded a few mg. of pure agnosteryl acetate (ϵ_{244} = 18,000; specific activity 40 c.p.m. per mg.). These light absorption values show that at the start the "isocholesteryl" acetate contained a total of 11 per cent of substances with the 7,9-diene structure which would include both agnosteryl acetate and dihydroagnosteryl acetate. The relative proportions of these two components cannot be ascertained, but it may be assumed for the present purpose that they occur in equal amounts. Hydrogenation data obtained with the "isocholesteryl" acetate used in this experiment indicate the presence of a total of 60 per cent material having an unsaturated side chain. Hence, if 5.5 per cent of the total mixture is agnosteryl acetate, the lanosteryl acetate comprises 54.5 per cent, and since the dihydro compounds contain no more than 5 per cent of the total radioactivity, the specific activity of lanosterol may be calculated to be 83 c.p.m. per mg.[4] or slightly more than twice that of the agnosterol.

It will be noted from Fig. 4 that during the enrichment of agnosteryl acetate on fractional crystallization the specific activity does not decline steadily but undergoes a marked increase during the initial steps. In accounting for this effect it must be remembered that "isocholesterol" is a mixture of four components. The first stages of crystallization of the acetates could result in the progressive removal of the dihydro compounds which, though present to the extent of 40 per cent of the total weight, account for no more than 5 per cent of the total radioactivity.

In the initial experiments carrier "isocholesterol," the only source of lanosterol then available to us, was added to the homogenates prior to incubation, for the purpose of trapping labeled lanosterol. The success of this procedure in producing a marked enhancement of the acetate incorporation into the "isocholesterol" fraction has already been noted. However, when lanosterol itself was used subsequently for the same purpose, it failed to reproduce the results obtained with "isocholesterol." Negative

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[4] If the value assigned to the agnosterol content of the original mixture is varied from 1 to 11 per cent, the calculated value for the specific activity of lanosterol would vary but slightly, namely from 86 to 80 c.p.m. per mg.

results were also obtained with dihydrolanosterol and agnosterol (Fig. 3). On the other hand, a "synthetic isocholesterol," reconstituted from the four known components in the proportions in which they seemed most likely to occur in our natural "isocholesterol," duplicated the results given by the natural mixture. It thus seems improbable that the enhancement is due to some hitherto unrecognized component of natural "isocholesterol," and this conclusion is supported by the finding that suspensions containing both lanosterol and agnosterol give a degree of enhancement comparable to that obtained with the four-component mixture. No explanation can at present be given for the synergistic action of lanosterol and agnosterol, but it is worth noting that these are the only two members of the "isocholesterol" group of sterols which became significantly labeled in the system studied. Further experiments to investigate the effect of other combinations of wool fat sterols are in progress.

Evidence for the conversion of lanosterol to cholesterol is presented in the succeeding paper (5). As an intermediate in cholesterol biogenesis, lanosterol would be expected to show the same distribution pattern of the carboxyl carbons of acetate as cholesterol itself. The isopropyl moiety of lanosterol isolated from the present experiments had a specific activity of 34 c.p.m., in reasonable agreement with the predicted value. A value in accord with current theory has also been obtained for carbon atom 11 of the C_{30} structure,[1] and hence it is probable that the carboxyl carbon atoms of acetate are distributed in lanosterol as they are in cholesterol.

SUMMARY

The unsaponifiable fraction of rat liver obtained on incubation of the homogenized tissue with radioactive acetate has been shown to contain two radioactive C_{30} sterols, lanosterol and agnosterol. When carrier "isocholesterol" is present during incubation, a much greater proportion of the radioactivity in the unsaponifiable fraction can subsequently be isolated in the lanosterol fraction.

The identity of the labeled products with lanosterol and agnosterol was established by preparation of several derivatives characteristic for this type of sterol. In the course of these transformations the C^{14} content of the C_{30} sterols remained unaltered.

A markedly increased yield of labeled lanosterol can be obtained by the incubation of liver homogenates in the presence of either "isocholesterol" from wool fat or of a synthetic isocholesterol reconstituted from the four pure components known to occur in the natural mixture. This enhancing effect is also shown by a mixture of lanosterol and agnosterol, but not by any single constituent of "isocholesterol."

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THE BIOLOGICAL CONVERSION OF LANOSTEROL
TO CHOLESTEROL*

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Lanosterol and agnosterol, two of the four known C_{30} sterols which have
so far been isolated from animal sources, have been shown by experiments
described in the preceding paper (1) to be synthesized from acetate by rat
liver homogenates. Attention has also been drawn to the possible rôle
of lanosterol or of related C_{30} sterols as precursors of cholesterol (2). Ex-
periments are now described in which biologically labeled lanosterol is
converted to cholesterol in rat liver homogenates.

EXPERIMENTAL

The materials and the methods of preparing rat liver homogenates were
those described in the preceding paper (1). For radioactivity measure-
ments, infinitely thin samples were plated and counted in a gas flow counter.

C^{14}-*Lanosterol*—Following the technique described previously, labeled
lanosterol was obtained by incubating emulsified "isocholesterol" with rat
liver homogenates containing acetate-1-C^{14}. This material, having 24,000
c.p.m. per mg., was diluted 20-fold by addition of pure lanosterol and re-
crystallized twice, giving lanosterol, m.p. 140–141°, with 1300 c.p.m. per
mg. (calculated 1200 c.p.m. per mg.). Chemical conversion of labeled
lanosterol of a comparable degree of purity to various typical derivatives
has been shown in the preceding paper (1) to take place without loss of
radioactivity. Before further use, the sterol was subjected to careful
"washing out" chromatography with pure non-radioactive cholesterol.
On recovery from the chromatogram the cholesterol fraction gave no de-
tectable radioactivity, thus eliminating cholesterol as a radioactive con-
taminant of the labeled lanosterol. Light absorption measurements in
the 244 mμ region indicated the presence of not more than 2 per cent of
substances having the 7,9-diene system (agnosterol and dihydroagnos-
terol). An exact value for the percentage of these substances could not be
obtained because of the necessarily high concentration of the solution used
for optical measurements.

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the American Society of Biological Chemists, San Francisco, April 12–15, 1955.

Conversion of Lanosterol to Cholesterol—12.0 mg. of C^{14}-lanosterol (1300 c.p.m. per mg., a total of 15,600 c.p.m.) were emulsified by sonic oscillation with 2.5 ml. of Bucher phosphate buffer containing 0.5 per cent bovine serum albumin as described previously (1), and the emulsion added to 40 ml. of rat liver homogenate together with 26 mg. of diphosphopyridine nucleotide. The mixture was divided into 2.5 ml. aliquots and incubated at 37° for $4\frac{1}{2}$ hours in an atmosphere of oxygen. The homogenates were then saponified with potassium hydroxide for 2 hours at 70° and the unsaponifiable material extracted with petroleum ether. The total activity of the unsaponifiable fraction was 15,480 c.p.m., a nearly quantitative recovery of the radioactivity originally added. Only 60 c.p.m. were found in the acidic fraction. Chromatography on a column of 10 gm. of alumina gave the fractions in Table I.

The cholesterol fractions (Fractions 10 to 12), when combined, weighed 11.4 mg. and had a specific activity of 36 c.p.m. per mg. This activity was retained through the purification procedures described below. The total activity of the cholesterol fractions in Table I corresponds to a conversion of 2.6 per cent of the added lanosterol. The data from this and two other experiments in which labeled lanosterol was added to the homogenate in lower concentrations are given in Table II. In all three experiments the major part of the radioactivity (60 to 70 per cent) was recovered as apparently unchanged lanosterol. Also a substantial fraction of the total radioactivity was invariably present in the form of compounds more polar than cholesterol.

The cholesterol fraction from Experiment 1 was diluted by addition of 11.4 mg. of non-radioactive cholesterol and crystallized to give 13.3 mg. of plates which melted at 148–149°, and had 18 c.p.m. per mg. There was therefore no loss of activity on crystallization. The product was now converted to the dibromide and the cholesterol, regenerated by debromination with zinc dust, and was recrystallized to give plates, m.p. 149–150°. This material gave 17 c.p.m. per mg.

The following procedure was designed to eliminate the possibility of contamination by compounds of higher specific activity, having double bonds in the 7,8, 8,9, 9,11, or 8,14 position. Cholesterol from Experiments 2 and 3 (Table II) was mixed with the purified cholesterol from Experiment 1 and the combined material (21 mg., 10.0 c.p.m. per mg.) was first acetylated, and then hydrogenated in neutral ethyl acetate. Under these conditions, nuclear double bonds in the region of the B,C ring junctions are not reduced or isomerized; double bonds in the 5,6 position, as in cholesteryl acetate, however, are hydrogenated. To transform any remaining unsaturated steroids to the corresponding epoxides, the hydrogenation product was treated with a large excess of permonophthalic acid

TABLE I

Chromatography of Unsaponifiable Fraction from Liver Homogenates Incubated with C^{14}-Lanosterol

Fraction No.	Solvent		Volume	Eluate residue	Total activity
			ml.		*c.p.m.*
1	Benzene		20	Trace yellow gum	15
2	Ether-benzene	1:99	20		10
3	"	1:99	20		3
4	"	1:99	60		0
5	"	1:99	20		15
6	"	1:99	120	White solid, 8.9 mg.; m.p. 135–140°	8170
7	"	2:98	20	Trace white solid	76
8	"	2:98	40	" gum	54
9	"	3:97	60		18
10	"	5:95	20	Trace solid	12
11	"	5:95	60	White " 5 mg.; m.p. 144–148°	174
12	"	1:9	85	White solid 6 mg.; m.p. 144–148°	202
13	"	1:9	40	Trace gum	24
14	"	1:4	40		64
15	"	1:1	40		57
16	"	1:1	20	Trace crystalline material	71
17	"	1:1	60		83
18	Ether		20		23
19	"		20	Trace solid	137
20	Ether-methanol 1:1		40	Gums	839

in ethereal solution for 4 days at room temperature, and subsequently re-isolated after extraction with alkali. The recovered material was now treated with an excess of lithium aluminum hydride in refluxing tetrahydro-

TABLE II

Conversion of C^{14}-Lanosterol to Cholesterol

Experiment No.	Lanosterol added, mg. per ml. homogenate	Lanosterol added Total activity, c.p.m.	Lanosterol recovered	Cholesterol Total activity, c.p.m.	Per cent conversion*	
					Calculation A	Calculation B
1	0.3	15,600	9500	410	2.6	6.9
2	0.025	1,300	890	104	8	25
3	0.0063	325	227	62	19	63

* Calculation A, per cent of added lanosterol converted to cholesterol; Calculation B, per cent of "utilized" lanosterol (lanosterol added-lanosterol recovered) converted to cholesterol.

furan for 50 minutes, with the object of reducing any epoxides to hydroxylated derivatives. The reaction mixture was distributed between ether and the minimum of dilute sulfuric acid required to clarify the aqueous phase. After washing with bicarbonate solution and water, the ether layer was evaporated to dryness *in vacuo* to give a white crystalline residue which was chromatographed on 1.5 gm. of alumina. Ether-benzene (2:98) eluted 15 mg. of solid material from which cholestanol, m.p. 141–142°, having 10.8 c.p.m. per mg., was obtained on crystallization from methanol.

DISCUSSION

The results of experiments in which biologically labeled lanosterol was tested as a precursor of cholesterol are given in Table II. In all three experiments a significant conversion was found to take place. The bulk of the activity, however, was recovered as unchanged lanosterol, presumably because the lanosterol suspension is inhomogeneous and contains particles too large to enter into the reaction. When the concentration of labeled lanosterol in the homogenates was progressively reduced, the percentage of radioactivity recovered in the form of cholesterol was markedly increased, though the total quantity converted was greater at higher concentrations of lanosterol. The efficiency of the conversion is perhaps more significantly represented by the values in the last column of Table II. These represent the activity recovered in cholesterol in per cent of the lanosterol *utilized*. On this basis it is calculated that 63 per cent of the metabolized lanosterol in Experiment 3, Table II, is converted to cholesterol.

It is of interest to compare the rate of this conversion with the rate of over-all synthesis of cholesterol from acetate in the same homogenate system. Calculations based on the specific activities of the precursor acetate and of the cholesterol isolated from incubation experiments carried out in this laboratory indicate that cholesterol is synthesized at the rate of approximately 5 γ per ml. of homogenate during a 5 hour period. According to Table II, the quantities of cholesterol derived from lanosterol under comparable conditions in Experiments 1, 2, and 3 were 7.8, 2, and 1.2 γ, respectively. Hence the rate of transformation of lanosterol to cholesterol is of the same order as the rate of the over-all synthesis.

The labeled lanosterol which served as a precursor of cholesterol in the present experiments was obtained by diluting a small quantity of biologically labeled "isocholesterol" with non-radioactive lanosterol. For the interpretation of the present results, it is therefore necessary to consider the possible contribution of C^{14} by components of "isocholesterol" other than lanosterol itself. According to the evidence discussed in the preceding paper (1), the two C_{30} sterols having saturated side chains, dihydrolanosterol and dihydroagnosterol, contribute at most 5 per cent of the radio-

activity in "isocholesterol" and have a low specific activity. On the other hand, agnosterol, separated from the biologically labeled "isocholesterol," was shown to have a specific activity equal to 80 per cent of that of the "isocholesterol." The proportion of agnosterol in "isocholesterol," although not known with certainty, may be assumed to be 5.5 per cent, or one-half of the total content (11 per cent) of substances having the 7,9-diene system as measured by the intensity of light absorption at 244 mμ. The proportion of the total radioactivity in the biologically labeled material which is attributable to agnosterol may therefore be calculated in the following way: 5.1 mg. of labeled "isocholesterol" contained a total of 122,000 c.p.m., or a specific activity of 24,000 c.p.m. per mg.; hence, the specific activity of agnosterol = 24,000 $\times$ 0.8 = 19,200 c.p.m. per mg. Since the total quantity of agnosterol is 5.1 $\times$ (5.5/100) = 0.28 mg., its total activity is 19,200 $\times$ 0.28 = 5380 c.p.m., and hence the percentage of the total radioactivity present as agnosterol is (5380 $\times$ 100)/122,000 or 4.4 per cent.

The labeled "isocholesterol," having 11 per cent 7,9-diene components, was diluted 20-fold with non-isotopic lanosterol which in itself contained 1.5 per cent of such dienes. After crystallization of the mixture, these compounds comprised 2 per cent of the product, as would be expected if no change in the proportions of the components occurred on crystallization. Hence, it can be assumed that, in the dilution and crystallization of labeled "isocholesterol" with lanosterol, the percentage contribution of agnosterol to the total radioactivity remains unchanged, namely 4.4 per cent. Assuming that, in the course of these procedures, the proportion of dihydro compounds also remained unchanged, then a total of 4.4 plus 5, or 9.4 per cent of the C^{14} content of the mixture, could have been present in substances other than lanosterol. In one out of the three experiments recorded in Table II (Experiment 3), 19 per cent of the substrate was converted to cholesterol, and hence lanosterol must be the principal single source of C^{14} for cholesterol.

The identity of the bulk of the radioactive product with cholesterol is demonstrated by the fact that its specific activity underwent no diminution on crystallization and purification in the usual way via the dibromide. It was considered possible, however, that at least a part of the product appearing in the cholesterol fraction could consist of sterols having double bonds in the same region of the nucleus as in lanosterol or agnosterol. It was therefore necessary to attempt some form of purification procedure for eliminating specifically such contaminants. The method employed was designed to convert cholesterol to cholestanol, while converting any compounds with double bonds in the 7,8, 8,9, 9,11, and 8,14 postions to more polar derivatives which would be readily separable by chromatog-

raphy.   In view of the retention of the original level of activity during conversion to cholestanol, it may be safely concluded that the C^{14} content of the cholesterol fraction is principally due to cholesterol itself.   The presence of companion substances with approximately equal specific activity cannot of course be ruled out on the basis of these purification methods.

While it is clear from the results of the present investigation that lanosterol can be a precursor of cholesterol, it does not follow that this transformation is a necessary event in the normal course of steroid biogenesis. Experiments with crude tissue preparations of the type used are inherently inconclusive in this respect.   Some evidence has been obtained, however, which is consistent with the view that lanosterol is a normal precursor of cholesterol.   The intestinal tract tissue of rats killed shortly after injection of acetate-C^{14} was found to contain lanosterol of specific activity several times higher than that of the cholesterol ioslated in the same experiments.[1] At longer time intervals after injection, the lanosterol fractions still contained C^{14}, but their specific activities were now relatively low compared

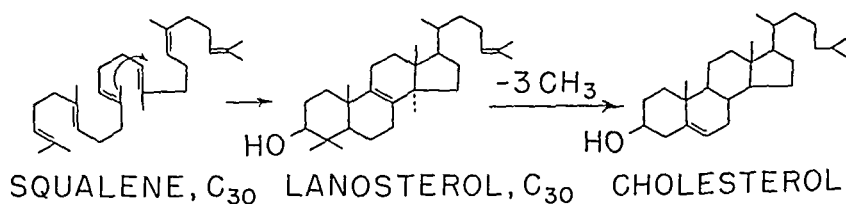SQUALENE, C_{30} LANOSTEROL, C_{30} CHOLESTEROL

Fig. 1

to those of cholesterol.   According to Schwenk et al. (3, 4), the cholesterol synthesized from acetate in similar experiments of short duration or in liver perfusions is accompanied by "high counting" materials which can serve as precursors of cholesterol.   It seems possible from the above results that, in part at least, the activity in these so far unidentified substances may be due to lanosterol.

Structural reasons make lanosterol particularly attractive as an intermediate in the conversion of squalene to cholesterol.   On the one hand, the constitution of lanosterol is suggestive of an origin from a triterpenoid precursor by cyclization; on the other hand, lanosterol shares with the steroids the tetracyclic ring system and can in fact be designated as a 4,4',14-trimethylcholestadienol.   It was on the basis of these relationships that the cyclization scheme given in Fig. 1 was proposed (2).   Moreover, the cyclization of squalene to lanosterol has been rationalized in terms of ionic mechanisms by the suggestion of Ruzicka (5) that, subsequent to an attack by a cationic reagent, the cyclization of the triterpene proceeds synchronously to lanosterol without stabilization of intermediates.   The distribution of the carbon atoms of acetic acid in biosynthetic squalene reported by

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[1] Unpublished experiments, carried out by P. Schneider in this laboratory.

Cornforth and Popják (6) and particularly the origin of C_7 and C_{13} (7, 2) of cholesterol from methyl carbons of acetate have until now been the most compelling evidence for the postulated mechanism of steroid biogenesis. That squalene itself is a direct precursor of the steroids, though in accord with all the experimental data, has remained a tentative conclusion because squalene is as yet not obtainable by organic synthesis, and because the identity of biologically labeled squalene has not been rigorously established. With the demonstration that lanosterol is synthesized in liver tissue and is efficiently converted to cholesterol, the squalene hypothesis has been considerably strengthened since squalene alone among naturally occurring compounds satisfactorily explains the origin of lanosterol and the rôle played by a C_{30} sterol in the biosynthesis of cholesterol.

SUMMARY

The conversion of lanosterol to cholesterol in rat liver homogenates has been demonstrated with the aid of biologically labeled lanosterol.

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DISTRIBUTION OF RADIOACTIVE CARBON DIOXIDE INCORPORATED INTO RAT LIVER GLYCOGEN*

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It has been demonstrated that $C^{14}O_2$ administered to fasted rats (2) or incubated with liver slices from fasted animals (3) yields glycogen which is labeled predominantly in carbon atoms 3 and 4. These findings are consistent with the interpretation that CO_2 incorporation into glycogen proceeds by a reversal of the anaerobic glycolytic pathway (4). However, during the past several years it has become evident that pathways other than the Embden-Meyerhof scheme may play a rôle in carbohydrate metabolism. It has previously been shown with liver enzyme preparations that pentose phosphate-2,3-C^{14}, which may arise by the oxidation of hexose monophosphate-3,4-C^{14}, yields glucose 6-phosphate labeled in positions 1 to 4 (5). If these reactions were to occur in intact liver, isotope originally incorporated into the 3,4 position of glucose 6-phosphate would be expected to become redistributed into positions 1 and 2. While evidence for the preferential formation of CO_2 from glucose C-1 by liver slices has been reported by Bloom, Stetten, and Stetten (6–8) and confirmed in a number of other laboratories (9–11), there is lack of agreement as to the quantitative importance of such non-glycolytic mechanisms (8, 11).

In general, studies of $C^{14}O_2$ incorporation into glycogen have been carried out with fasted animals. It has been demonstrated, however, that fasting markedly affects glycogen formation (12), glucose and pyruvate utilization (13), and fatty acid and CO_2 production (14). The present investigations were undertaken to ascertain whether the feeding of a high carbohydrate diet might lead to a distribution of tracer in glucose, which is different from that observed in studies with fasted animals. Liver slices from fasted and from fed rats were incubated with lactate in the presence of $C^{14}O_2$ and the glycogen was isolated and degraded. It was found that, while positions 3 and 4 were labeled equally when liver

* While this manuscript was in preparation, the authors learned of similar experiments with pyruvate-2-C^{14} carried out by Landau et al. (1).

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slices from fasted rats were employed, in almost every experiment with slices from fed animals carbon atom 4 had a higher specific activity than carbon atom 3. When glycerol was included in the medium, the distribution of tracer in glycogen from liver slices of fasted rats became similar to that obtained with tissue from fed animals. In most experiments, small amounts of isotope were incorporated into the remaining carbon atoms; usually positions 1 and 2 were more heavily labeled than positions 5 and 6.

Methods

Tissue Preparation—Male rats of the Sprague-Dawley strain, weighing between 150 and 225 gm., were used. For a 4 day period prior to the experiment, these rats were offered a diet containing 58 per cent glucose, 22 per cent casein, 6 per cent brewers' yeast, 6 per cent salt mixture (15), 2 per cent liver powder (Lederle), 4 per cent Cellu flour, and 2 per cent vitamins A and D in Wesson oil (14). In the studies with liver slices from fasted rats, all food was withdrawn for a 72 hour period prior to sacrifice. Both groups of rats were given water *ad libitum*. On this regimen, the fed rats gained between 10 and 24 gm. in weight and the fasted rats lost 25 to 40 gm. The rats were sacrificed by decapitation and the livers removed and placed in a slicing medium chilled over ice. The slicing media were identical with the incubation media, except that they contained no substrates. The tissue slices, prepared with a Stadie-Riggs slicer (16), were blotted free of excess liquid with filter paper and weighed on a torsion balance. In order to obtain an adequate amount of glycogen for degradation, a total of 4 gm. of liver slices was used in the experiments with fed rats and 6 gm. of liver slices in the experiments with fasted animals.

Incubation Procedure—The C^{14}-bicarbonate solution was prepared by absorbing $C^{14}O_2$ liberated from 1.0 mc. of $BaC^{14}O_3$ in a slight excess of 0.5 N NaOH or KOH for the experiments with Medium I or Medium II, respectively. The radioactive solution was adjusted to the phenolphthalein end-point with 0.2 N HCl. The final solutions contained 1 to 3×10^6 c.p.m. per ml., as determined by counting 0.02 ml. aliquots at infinite thinness.

The incubation vessels were 125 ml. Warburg type flasks. Each contained 2.0 ± 0.1 gm. of liver slices in a final volume of 21.5 ml. This consisted of 1 ml. of substrate solution, 0.5 ml. of the C^{14}-bicarbonate solution, and 20 ml. of a bicarbonate buffer solution previously saturated with a gas mixture containing 95 per cent O_2 and 5 per cent CO_2. Two bicarbonate buffer solutions with varying concentrations of Na^+ and K^+ were used. The ionic composition of Medium I was similar to the Krebs-Ringer bicarbonate buffer as described by Umbreit *et al.* (17), except that

the final Na^+ concentration was 158 m.eq. per liter, and the Cl^- concentration was 111.0 m.eq. per liter. Medium II contained 110 m.eq. of K^+ and no Na^+, and was similar to that described by Hastings *et al.* (18), except that the chloride concentration was 106 m.eq. per liter.

The substrate solutions contained either 600 μmoles of lactate or 300 μmoles of lactate plus 300 μmoles of glycerol, adjusted to pH 7.4. The flasks were placed in a 38° water bath and aerated for 10 minutes with the O_2-CO_2 gas mixture. The pH of the reaction mixture was 7.4 to 7.5. Following aeration, 0.5 ml. of C^{14}-bicarbonate solution was introduced into a side arm and tipped into the incubation medium. The flasks were incubated for 15 to 240 minutes.

Glycogen Degradation—Glycogen was isolated from the liver slices and purified to constant specific activity by the method of Stetten and Boxer (19). Glucose was obtained by hydrolysis of the glycogen with 1 N H_2SO_4 as described by Topper and Hastings (3). Reducing sugar was determined by the method of Somogyi (20).

Glucose was degraded by fermentation with *Leuconostoc mesenteroides*, which has been shown by Gunsalus and Gibbs (21) to yield CO_2, ethanol, and lactic acid. In the present experiments about 400 μmoles of glucose were fermented. The CO_2 (carbon atom 1) released during fermentation was absorbed in 2 N CO_2-free KOH. The ethanol (carbon atoms 2 and 3) was oxidized to acetic acid by heating for 2 hours at 90° with 0.5 gm. of potassium dichromate in 4 N sulfuric acid and the product degraded by the method of Phares (22). Lactic acid (carbon atoms 4, 5, and 6) was oxidized to CO_2 (carbon atom 4) and acetic acid (carbon atoms 5 and 6) with chromium trioxide in 1 N sulfuric acid (23). The acetic acid formed was degraded as before.

Radioactivity measurements were made with a gas flow counter (24). All CO_2 samples were converted to barium carbonate and counted at infinite thickness. The reliability of the degradation procedure was checked with samples of glucose-3,4-C^{14}, glucose-1-C^{14}, and uniformly labeled glucose-C^{14}. The results of these degradations are presented in Table I.

RESULTS AND DISCUSSION

With lactate as the only added substrate, 86 to 99 per cent of the total $C^{14}O_2$ incorporated into glycogen entered carbon atoms 3 and 4 (Tables II and III). With liver slices from fasted rats, these positions were essentially equally labeled (Experiments 1 through 3). However, in almost every experiment with liver slices from fed animals, carbon atom 4 had a higher specific activity than carbon atom 3 (Table III). The pattern of isotope distribution in the carbon chain of glycogen in the experiments

with either fasted or fed rat liver slices was similar whether the incubation medium contained a high Na^+ concentration (Experiments 1, 2, and 7 through 13) or no Na^+ and a high K^+ concentration (Experiments 3, 14, and 15).

TABLE I

Degradation of Glucose-3,4-C^{14}, Glucose-1-C^{14}, and Uniformly Labeled Glucose

Compound	Relative activities in glucose carbon atoms*					
	C-1	C-2	C-3	C-4	C-5	C-6
Glucose-3,4-C^{14}†.............	2.3	0.9	48.0	48.8 (41,400)	0.0	0.0
Glucose-1-C^{14}‡..............	99.4 (26,100)	0.2	0.2	0.0	0.2	0.0
Uniformly labeled glucose§...	16.9	15.9	16.6	17.2	17.5 (8590)	15.9

* The specific activities (counts per minute per millimole of carbon) of the carbon atoms indicated are given in parentheses.

† Glucose-3,4-C^{14} was obtained from the liver glycogen of fasted rats after intraperitoneal administration of $C^{14}O_2$ (2). The above data are in agreement with evidence for equal labeling in carbon atoms 3 and 4 obtained by other methods of degradation (3).

‡ Kindly supplied by Dr. H. S. Isbell, National Bureau of Standards.

§ Kindly supplied by Dr. M. Gibbs, Brookhaven National Laboratory.

TABLE II

Distribution of C^{14} in Glycogen of Liver Slices from Fasted Rats

Experiment No.	Incubation*			Per cent total activity†					
	Substrate	Medium	Duration	C-1	C-2	C-3	C-4	C-5	C-6
			min.						
1	Lactate	I	30	1.1	1.1	47.4	48.0 (4020)	1.2	1.2
2	"	I	120	1.8	1.0	47.6	48.6 (4140)	0.5	0.5
3	"	II	30	0.5	0.5	48.8	50.2 (810)	0.0	0.0
4	Glycerol + lactate	I	30	0.6	3.9	33.5	60.3 (1780)	0.3	1.4
5	" + "	I	30	2.4	4.1	29.5	56.8 (580)	4.0	3.2
6	" + "	I	30	1.1	1.9	32.0	63.5 (194)	1.1	0.4

* Substrate concentrations and composition of media as described in the text.

† The specific activities of the carbon atoms indicated are given in parentheses.

The appearance of label predominantly and symmetrically in carbon atoms 3 and 4 of glucose in the experiments with liver slices from fasted rats is in accord with previous observations (2, 3). This finding is consistent with the interpretation that, under these experimental conditions, carboxy-labeled phosphopyruvic acid, arising from $C^{14}O_2$ fixation into

dicarboxylic acids, contributes equally to carbon atoms 1 through 3 and 4 through 6 of glycogen.

The lower specific activity of carbon atom 3 in the liver glycogen of fed rats may be attributed to the presence in these tissues of unlabeled triose phosphate which would consist mainly of dihydroxyacetone phosphate, and which would condense with newly formed labeled glyceraldehyde 3-phosphate. The validity of this interpretation was tested by incubating liver slices from fasted rats with glycerol, a precursor of dihydroxyacetone phosphate, together with lactate and $C^{14}O_2$. This addition resulted in

TABLE III

Distribution of C^{14} in Glycogen of Liver Slices from Fed Rats

Experiment No.	Incubation*		Per cent total activity†					
	Medium	Duration	C-1	C-2	C-3	C-4	C-5	C-6
		min.						
7	I	15	4.5	0.5	43.2	50.5 (588)	0.8	0.5
8	I	20	0.5	0.5	41.1	56.4 (1020)	1.0	0.5
9	I	30	2.6	1.3	40.3	52.9 (1150)	2.4	0.5
10	I	60	2.1	0.4	41.7	54.5 (6840)	0.3	1.0
11	I	75	2.7	2.6	39.6	53.4 (1230)	1.3	0.4
12	I	180	2.3	4.6	39.7	50.6 (4310)	1.6	1.2
13	I	220	1.4	3.9	42.8	45.6 (7710)	4.1	2.2
14	II	15	1.1	2.4	41.6	54.2 (2170)	0.3	0.4
15	II	30	1.7	1.8	43.9	50.5 (3920)	1.1	0.9

* Substrate (lactate) concentration and composition of media as described in the text.

† The specific activities of the carbon atoms indicated are given in parentheses.

glycogen more heavily labeled in carbon atom 4 than carbon atom 3 (Experiments 4 and 5).

These findings complement those of Schambye et al. (25), who observed that the administration of glycerol-1-C^{14} to fasted rats resulted in glycogen which contained more C^{14} in carbon atom 3 than in 4. Recently, Dische and Rittenberg (26) reported the asymmetric labeling of glucose following the administration of phenylalanine-4-C^{14} to fasted rats. These earlier observations of others and the present findings of unequal labeling of carbon atoms 3 and 4 are compatible with the hypothesis that the incorporation of tracer into these positions of glucose is dependent on a balance between the reactions involving the isomerization of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, the condensation of these esters to fructose 1,6-diphosphate and its conversion to fructose 6-phosphate, and the formation of dihydroxyacetone phosphate from

glycerol via α-glycerophosphate. The fed rat has relatively large endogenous, hence unlabeled, sources of dihydroxyacetone phosphate precursors, i.e. glycogen and glycerophosphate, and a similar condition arises when rat liver slices from fasted animals are incubated with unlabeled glycerol. The present results suggest that, under these conditions, the condensation of glyceraldehyde 3-phosphate-1-C^{14} and dihydroxyacetone phosphate proceeds more rapidly than their isomerization. While this mechanism is not the only one by which $C^{14}O_2$ may label carbon atom 4 more heavily than carbon atom 3, it is likely that it makes the major contribution to this asymmetrical labeling.

It has previously been reported that traces of isotope from $C^{14}O_2$ are incorporated into positions 1, 2, 5, and 6 (27). In the present experiments these carbon atoms were labeled consistently with specific activities reaching 5 to 10 per cent of those found in carbon atoms 3 and 4 (Tables II and III). This result may reflect the operation of the pentose phosphate pathway (28). Other explanations for the incorporation of $C^{14}O_2$ into positions other than 3 and 4 are available. Thus a condensation of pyruvic acid and glyceraldehyde 3-phosphate, by a reversal of the reactions described by Entner and Doudoroff (29), would result in hexose monophosphate labeled in carbon atoms 1 and 4. $C^{14}O_2$ might also be incorporated into carbon atom 1 by fixation to pentose phosphate (30, 31). At the present time, however, there is no evidence for either of these processes in mammalian tissue.

It must be emphasized that the isotope incorporation into carbon atoms 1, 2, 5, and 6 was small and somewhat variable compared to the labeling of positions 3 and 4. It would thus appear that, although under the conditions of the present experiments several pathways are present in liver, the glycolytic mechanism plays the major rôle in the synthesis of glycogen.

SUMMARY

Liver slices from fasted and fed rats were incubated with $C^{14}O_2$ and lactate, and the glycogen was isolated and degraded. With slices from fasted rats, positions 3 and 4 of the glycogen were labeled equally, while with liver slices from fed animals the activity in carbon atom 4 was 15 to 35 per cent higher than in carbon atom 3. The amount of isotope incorporated into position 3 in the glycogen from slices from fasted animals was depressed when non-isotopic glycerol was added to the medium. Small but reproducible quantities of C^{14} also appeared in carbon atoms 1, 2, 5, and 6. Possible interpretations for these observations are discussed.

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FORMATION OF TESTOSTERONE GLUCURONIDE BY SURVIVING LIVER SLICES*

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Testosterone is known to be metabolized mainly to the saturated 17-keto-steroids, androsterone and etiocholanolone (1–5). Inasmuch as a number of steroid hormones and their metabolites are converted to conjugated glucuronides, testosterone may be expected to form a glucuronide in the organism. Since testosterone, either free or conjugated, has not been detected in urine, it seemed more profitable to search for testosterone glucuronide as a product of the metabolism of testosterone *in vitro*. Experimental data supporting the view of the existence of testosterone glucuronide are described in this paper.

Methods

The following provided the basis for the separate quantitative determination of testosterone glucuronide and testosterone present at the end of the period of incubation of testosterone with surviving liver slices.

Principle—Testosterone was quantitatively removed from the deproteinized incubation mixture by benzene extraction at pH 7.5, following which its glucuronide was extracted with ethyl acetate at pH 1.0. β-Glucuronidase hydrolysis of an aqueous solution of the glucuronides, thus extracted, released testosterone and glucuronic acid. The testosterone was determined spectrophotometrically as the thiosemicarbazide derivative (6) and the glucuronic acid by the microanalytical naphthoresorcinol method of Fishman and Green (7).

Reagents—Testosterone c.p. (a kind gift from The Upjohn Company) Krebs-Ringer bicarbonate and phosphate buffers (8), propylene glycol c.p. and thiosemicarbazide (Eastman Kodak), liver β-glucuronidase, Ketodase (Warner-Chilcott).

Experimental Design—Adult male rats (approximately 250 gm.) were killed by dislocating the cervical vertebrae. The livers were removed by

rapid dissection and cooled in ice, and then slices were cut free-hand with a razor blade. Each experiment was carried out in quadruplicate. The slices were suspended in Krebs-Ringer bicarbonate buffer at pH 7.4 in a total volume of 6.0 ml., 1.7 μmoles of testosterone in 0.2 ml. of propylene glycol having been added previously. The controls were set up in the absence of testosterone. The mixtures were stoppered and shaken for 2 hours in air at 38° in a Dubnoff shaking incubator. After this procedure, 1.7 μmoles of testosterone in 0.2 ml. of propylene glycol were added to the control flasks. The incubated mixture in each case was deproteinized by adding 1.0 ml. of 10 per cent $ZnSO_4$ plus 1.2 ml. of 0.5 N NaOH and 6.8 ml. of H_2O. The liver slices were removed immediately and rinsed with water and their weight determined after drying at 100°. The mixture remaining was centrifuged, and the supernatant solutions were pooled and processed as follows.

A 25 ml. aliquot of this mixture was extracted five times with 10 ml. portions of benzene. The aqueous phase was then made 2 N with respect to hydrochloric acid and extracted four times with 30 ml. volumes of ethyl acetate. The ethyl acetate was distilled under reduced pressure, and the residue was usually dissolved in 14 ml. of hot water and filtered. From 5 to 8.5 ml. of the filtrate, depending on the concentration of glucuronide expected, were incubated with 2500 Fishman units (9) of β-glucuronidase in a final volume of 10 ml. at pH 4.6 (0.1 M acetate buffer) overnight. This digest was deproteinized with $Zn(OH)_2$ and the liberated testosterone extracted by 10 ml. of benzene (vigorous shaking for 4 minutes). The aqueous phase contains the free glucuronic acid.

An aliquot (1 or 2 ml.) of the benzene extract was evaporated to dryness, and the product was treated with thiosemicarbazide in glacial acetic acid (6). The optical density was measured at 300 mμ, and the testosterone concentration was read from a previously established linear calibration curve relating testosterone concentration to optical density. Testosterone may also be determined by measuring its optical density in absolute methanol at 240 mμ. The absorption spectrum of testosterone in concentrated sulfuric acid was determined only in experiments designed to identify testosterone prepared by hydrolyzing its conjugate. Glucuronic acid liberated by β-glucuronidase was determined by the method of Fishman and Green (7).

In control experiments with liver tissue, 90 per cent of the added testosterone can be recovered, and hence the analytical figures for these have been increased by 10 per cent as a correction.

EXPERIMENTAL

Formation of Testosterone Glucuronide—Each of many control experiments in which testosterone was added to the incubation mixture *after* the

interval of incubation failed to produce steroid material which was ethyl acetate-extractable and which contained conjugated glucuronic acid. The product of the test runs invariably behaved as a glucuronide under the conditions of the naphthoresorcinol reaction designed for measuring glucuronide glucuronic acid (7). The liberation of free glucuronic acid from this conjugate by β-glucuronidase was paralleled by the disappearance of the glucuronide (7). Similarly, the rate of liberation of testosterone was of the
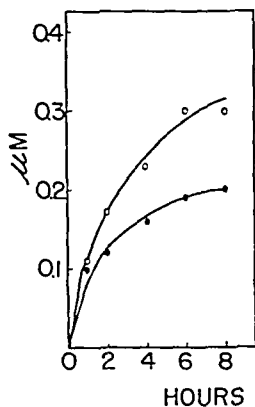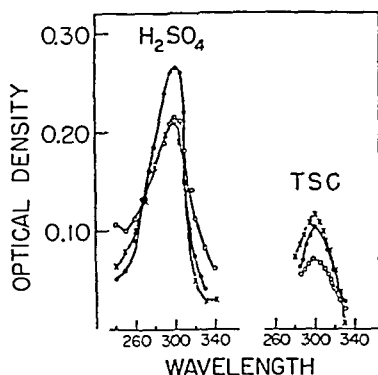
FIG. 1   FIG. 2

FIG. 1. Rate of liberation of testosterone ($\bullet$) and glucuronic acid ($\bigcirc$) from the product during β-glucuronidase hydrolysis. The reaction mixture contained conjugates corresponding to 0.3 μmole of glucuronide glucuronic acid and 0.3 ml. of enzyme (1500 Fishman units) in a total of 10 ml. of 0.1 M acetate buffer, pH 4.6. Intervals of incubation at 37° are indicated.

FIG. 2. Ultraviolet absorption spectra of steroid liberated by β-glucuronidase hydrolysis of the glucuronide. $\bullet$, testosterone; $\bigcirc$, product from one experiment; $\times$, product from a second experiment. The optical density was measured with a Beckman ultraviolet spectrophotometer. Conditions for sulfuric acid treatment were 30 minutes heating at 100° in concentrated H_2SO_4. The thiosemicarbazone derivative (T. S. C.) was prepared as described in the text.

same order as the rate of release of free glucuronic acid by β-glucuronidase (Fig. 1), and in some other experiments (Fig. 7) equimolar amounts of testosterone and glucuronic acid were liberated. The ultraviolet absorption spectrum of the steroid product of two different experiments (after β-glucuronidase hydrolysis) was identical to that of testosterone (Fig. 2).

The steroid product of a third experiment was subjected to paper chromatography according to the standard Bush procedure. A single zone which absorbed in the ultraviolet and gave a positive Zimmermann reaction was observed. This material had the same R_F value as the reference standard, testosterone, run simultaneously. Material obtained in a similar fashion from control flasks was negative for testosterone. This identifica-

tion study was carried out through the kindness of Dr. L. L. Engel of the Massachusetts General Hospital.

The steroid glucuronide was hydrolyzed by β-glucuronidase at an optimal pH of 4.5, a value typical of pH optima of substrates for this particular enzyme (Fig. 3) (10).

The ultraviolet absorption spectrum of testosterone, whether measured in H_2SO_4 *or in methanol or as the thiosemicarbazone derivative in glacial*
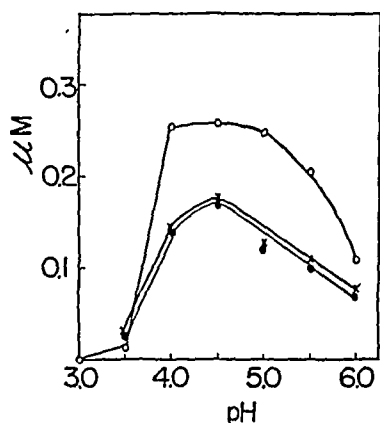

FIG. 3   FIG. 4

FIG. 3. Effect of pH on hydrolysis of glucuronide by β-glucuronidase. Each reaction mixture contained 0.46 μmole of substrate and 0.3 ml. of 1500 Fishman units of β-glucuronidase in a total of 10 ml. of 0.1 M acetate buffer of a given pH. Incubation at 37° for 4 hours. ●, testosterone (thiosemicarbazone); ✕, testosterone (in methanol); ○, hydrolyzable glucuronic acid.
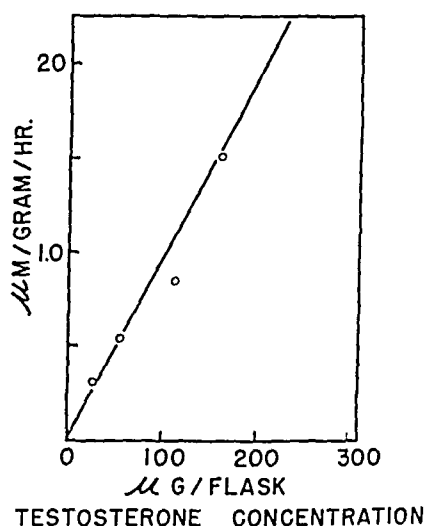
FIG. 4. Testosterone concentration and glucuronide formation on conjugation. The reaction mixtures each contained around 100 mg. of fresh liver slices, varying amounts of testosterone, and Krebs bicarbonate buffer (final volume 6 ml.). These were incubated at 37° for 2 hours in a Dubnoff shaking incubator. Hydrolyzable testosterone was measured.

acetic acid, is due to the 3-keto, Δ^4 configuration of the molecule. In the event of a reduction of either or both the 3-keto and Δ^4 linkages, absorption in the ultraviolet region would be changed or abolished. Accordingly, the differences noted (*e.g.*, Figs. 3, 5, 8) in the figures between micromoles of conjugate based on glucuronic acid analyses and micromoles of conjugate determined from hydrolyzable testosterone are due no doubt to non-testosterone glucuronides.

Effect of Testosterone Concentration on Glucuronide Formation—Fig. 4 illustrates the influence of testosterone concentration in the incubation medium upon the rate of glucuronic acid conjugation. A linear relationship was noted, conjugation being measured in the presence of as little as

25 γ of testosterone in the digest. A maximal synthesis occurred at a concentration of 500 γ per flask, the rate falling with concentrations of steroid greater than this (data not shown in Fig. 4).

Effect of pH of Incubation Mixture on Testosterone Glucuronide Synthesis—According to Fig. 5, the optimal pH for synthesis is 7.4. It should be noted that the rate of synthesis is greater when the medium contains Krebs-Ringer
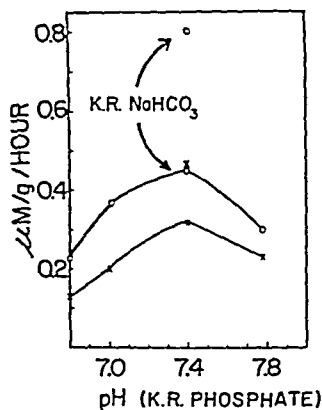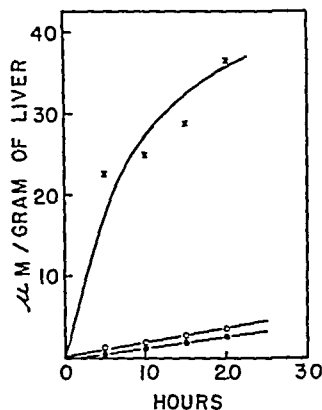

FIG. 5. Effect of pH on testosterone conjugation. The reaction mixture contains Krebs-Ringer phosphate buffer at different pH values as indicated. ×, testosterone; O, glucuronic acid.

FIG. 6. Relative rates of disappearance and of glucuronidation of testosterone in the presence of rat liver. Incubation mixtures were constructed as described in the text and deproteinized at the time intervals indicated. ×, disappearance of testosterone based on testosterone analysis of the first benzene extract; these values are corrected to 1 gm. of liver in the zero time incubation mixture; O, steroid glucuronide content based on analysis for hydrolyzable glucuronic acid; ●, testosterone glucuronide content based on hydrolyzable testosterone.

bicarbonate rather than phosphate buffer, a finding resembling the observations of others who used the *o*-aminophenol-liver slice system (11).

Time Relationships Observed in Rates of Testosterone Disappearance and Testosterone Glucuronide Synthesis—Testosterone, as measured by ultraviolet absorption spectrometry, was metabolized rapidly, according to Fig. 6, whereas a linear rate of glucuronide synthesis was noted over the time period of the experiment (2 hours). The percentage of testosterone being converted to glucuronide was only a fraction of the amount of the steroid disappearing (about 5 to 10 per cent). However, at the end of 2 hours, a major portion of the testosterone remaining in the digest was being conjugated with glucuronic acid.

Effect of Liver Concentration—As shown in Fig. 7, the highest rate of tes-
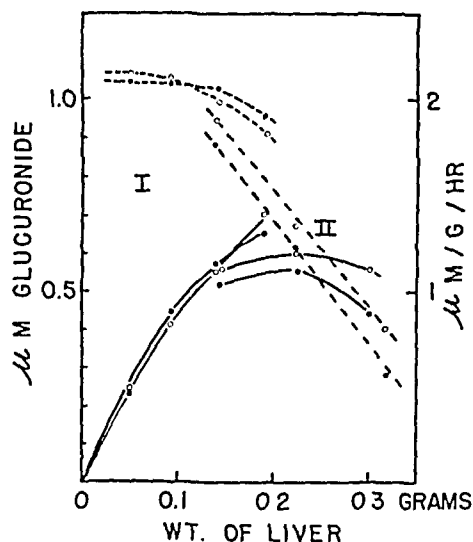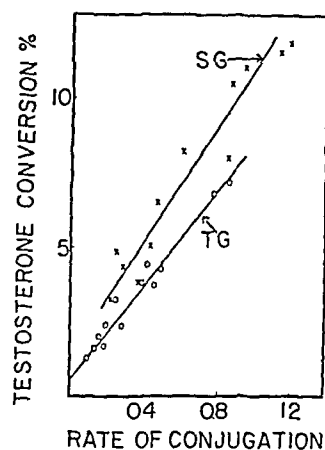
FIG. 7

FIG. 8

FIG. 7. Effect of liver concentration. Slices from Liver I provided the material from which the curves on the left region of the figure were obtained; slices from Liver II, the curves on the right portion of the figure. ●, testosterone glucuronide, based on analysis for hydrolyzable testosterone; ○, steroid glucuronide, based on analysis for hydrolyzable glucuronic acid.

FIG. 8. Rate of glucuronide synthesis as a function of the percentage of total testosterone conjugated. ✕, steroid glucuronide synthesis based on hydrolyzable glucuronic acid; ○, testosterone glucuronide synthesis based on hydrolyzable testosterone; rate of conjugation as micromoles per gm. per hour.

TABLE I

Experiments on Testosterone Conjugation with Livers of Six Animal Species

Species	Formation of steroid glucuronide based on hydrolyzable glucuronic acid		Formation of testosterone glucuronide based on hydrolyzable testosterone	
	*per cent**	*μmoles per gm per hr.*	*per cent**	*μmoles per gm per hr*
Hamster 1	5.50	0.276	1.55	0.264
" 2	8 05	0.402	1.57	0.267
Mouse 1	4.03	0.655	2.05	0.211
" 2	5.10	0.650	2.95	0.251
Rabbit 1	1.59	0.186	0.75	0.088
" 2	7.10	0.943	2.95	0.392
Guinea pig 1	6.75	0.326	2.04	0.114
" " 2	10.20	0.562	3.45	0.202
Dog 1	19.25	1.535	15.45	1.276
" 2	21.10	2.270	16.80	1.770
Rat 1	11.70	1.185	6.70	0.770
" 2	13.20	1.320	8.00	0.845

* Per cent of testosterone originally present in the incubation mixture which is conjugated in 2 hours.

tosterone conjugation occurred with small amounts of liver, all of the hydrolyzable glucuronic acid being accounted for by β-glucuronidase-hydrolyzable testosterone. The absorption spectrum in methanol of the unhydrolyzed product exhibited a maximum at 240 mμ identical to testosterone, indicating therefore, that glucuronic acid is not attached to ring A of the steroid.

Variability of Rate of Testosterone Glucuronide Formation in Rat Liver— In Fig. 8 are collected data obtained in thirteen different experiments with rat livers. The increase in the rate and extent of steroid glucuronide formation may be explained by an enhanced synthesis of testosterone glucuronide.

Testosterone Glucuronide Formation by Livers of Several Animal Species— From Table I it is seen that the livers of all of the animal species studied accomplished the formation of testosterone glucuronide, dog liver being apparently the most efficient of the group. The metabolic conjugation of testosterone with glucuronic acid is probably a characteristic of mammalian liver.

DISCUSSION

The experimental evidence supports the view that surviving slices of rat and other mammalian species accomplish the metabolic conjugation of testosterone, yielding testosterone glucuronide. Thus, in the presence of testosterone, a substance extractable from acid solution by ethyl acetate is produced, which gives a reaction for glucuronide glucuronic acid. β-Glucuronidase hydrolyzed this material (7) (also this paper), yielding steroid material and glucuronic acid. Most of this steroid is indistinguishable from testosterone according to measurements of its ultraviolet absorption spectrum whether in methanol or sulfuric acid or as the thiosemicarbazone derivative in glacial acetic acid and on the basis of paper chromatography. Often the figures for hydrolyzable glucuronic acid and testosterone agree within experimental error, as in Fig. 7.

Some of the favorable conditions for observing the conjugation have been established. These are optimal pH of the incubation, the most suitable concentration of testosterone, and the desirability of employing small amounts of liver tissue in the incubation.

Quantitatively, the amount of testosterone converted to the glucuronide is relatively small and for this reason may have been overlooked by previous workers (12–14).

Interpretation of Rôle of Testosterone Glucuronide—Since the formation of this substance can be detected in surviving liver slices in microgram amounts, it is entirely possible that testosterone glucuronide may be produced *in vivo*. The question is whether or not this conjugate is an obliga-

tory step in the metabolism of testosterone by liver and, if not, what is its biochemical significance.

The rather considerable disappearance of testosterone (ultraviolet absorption) from the incubation mixture at a time when the conjugation rate is low strongly suggests that in surviving liver slices glucuronide formation may not be an obligatory step in its metabolism. Furthermore, in order to account for the formation *in vivo* of androsterone and etiocholanolone from testosterone glucuronide, one would have to postulate, in addition to reduction of ring A of the steroid, some mechanism for producing the 17-keto group from the conjugated 17-hydroxyl. Evidence for such a mechanism (perhaps, involving simultaneous oxidation and hydrolysis) is lacking.

However, formation of testosterone glucuronide may be a means of preserving the hormone in its passage from the testis through the circulation and the liver on to its target organs. Certainly, the capacity of the liver, and other tissues, such as prostate and skin (14), to oxidize testosterone is sufficiently great to destroy the small amounts of testosterone which are supposedly produced endogenously. Thus, a need for a protective conjugation may be visualized. The possibility of conjugates of steroid hormones being the materials utilized by the target organs has been mentioned previously (15).

Testosterone glucuronide should now be added to a growing list of hormone glucuronides which includes the glucuronides of estriol, estrone, thyroxine, and epinephrine.

SUMMARY

Upon incubating testosterone with surviving slices of liver taken from six different animal species, a product with the properties of testosterone glucuronide is formed. Depending on the species and amount of liver used, from 1 to 20 per cent of the testosterone is converted into material which is extractable from acid solution by ethyl acetate, gives a reaction for glucuronide glucuronic acid, and, on β-glucuronidase hydrolysis, yields in nearly equimolar amounts glucuronic acid and a steroid which is indistinguishable spectrophotometrically and chromatographically from testosterone. The optimal pH of hydrolysis of this product is 4.5.

Experimental conditions have been defined which yield optimal synthesis of the glucuronide. These include the H^+ concentration of the incubation mixture, tissue weight, testosterone concentration, and time.

This is another example of the formation of hormone glucuronides, the significance of which remains to be investigated.

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ENZYMATIC CARBOXYL ACTIVATION OF AMINO ACIDS*

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Previous work in this laboratory (1–3) had revealed that the incorporation of C^{14}-labeled amino acids into the protein of rat liver microsome fraction was dependent upon ATP[1] and enzymatic components of the soluble protein fraction. Part of this enzymatic requirement was accounted for by enzymes which would generate ATP from a precursor such as phosphocreatine, phosphopyruvate, or phosphoglycerate. It was apparent, however, that, after fortification of the incorporation system with the precursors and the appropriate ATP generating enzymes, heat-labile, non-dialyzable components of the soluble fraction were still required.

It therefore seemed reasonable to subject to experimental test the possibility that a mechanism for amino acid activation by ATP resides in this soluble protein fraction. Preliminary results of such a study (4) revealed that the dialyzed soluble protein fraction of rat liver catalyzes an exchange of PP^{32} with ATP which is enhanced several fold by the addition of a group of pure L-amino acids. The microsome fraction also catalyzes a PP^{32} exchange which is not, however, influenced by amino acids. It was found, furthermore, that AMP fails both to inhibit the amino acid-dependent exchange and to exchange with ATP (by using C^{14}-labeled AMP). These results suggested that the amino acids were being activated as an amino acyl $\sim$ AMP compound. This possibility was given further support by the finding that α-amino hydroxamic acids are formed in the presence of high hydroxylamine concentrations, with concomitant loss of ATP. Since the amino acids did not cause a net splitting of ATP unless hydroxylamine was present, it was proposed that the amino acyl $\sim$ AMP is bound on the enzyme surface and dissociates from the enzyme to only a small extent if at all. The L-amino acid effect on exchange and on hydroxamic acid formation was additive with different amino acids, and D-amino acids were inert in the system.

These findings suggested a hypothesis for amino acid activation in animal tissue (4), which has since received further support by partial purification of the system and a more detailed analysis of its properties. The present paper deals with these extensions of the earlier work.

<div align="center">EXPERIMENTAL</div>

Enzyme Preparations—The livers of young (80 to 100 gm.) Wistar strain rats were used throughout. All operations were performed at 0°. 5 gm. of liver were homogenized with 7 ml. of 0.05 M KCl and, after this homogenate was diluted with 1 or 2 volumes of 0.05 M KCl, it was spun at 105,000 × g for 50 minutes in a Spinco preparative ultracentrifuge.

The soluble protein fraction of rat liver was drawn off with a syringe and the pH brought to 5.3 to 5.1 by the dropwise addition of 0.1 N HCl with constant stirring. The resultant precipitate was packed by centrifuging at 10,000 × g for 5 minutes. For certain experiments in which it was desirable to have minimal base-line exchange or hydroxamic acid formation, the soluble protein fraction was first diluted 6-fold with 0.05 M KCl and subjected to the same isoelectric precipitation, and the precipitate was washed once with a smaller volume of KCl. In all cases the precipitate was finally resuspended by homogenization in 0.1 M Tris buffer, pH 7.6 (to give a final protein concentration of about 30 mg. per ml.), recentrifuged at 15,000 × g, and the undissolved material discarded. Protein concentration was determined turbidimetrically with trichloroacetic acid. (0.5 to 3 mg. of protein in 7.0 ml. of 3 per cent trichloroacetic acid was mixed thoroughly and read at 540 mμ in a Coleman spectrophotometer 30 seconds later. Standard calibration was carried out with known quantities of soluble protein fraction protein.) The preparation is referred to throughout as the pH 5 enzyme.

The fraction active for methionine hydroxamic acid formation was derived from the soluble protein fraction by treatment with saturated ammonium sulfate solution. The fraction precipitating between 35 and 45 per cent saturation was collected by centrifugation, resuspended in 0.005 M phosphate, pH 7.0, to give a final protein concentration of about 30 mg. per ml., and dialyzed against 200 times the volume of the same concentration of phosphate for 18 hours.

For the incorporation of C^{14}-amino acids into protein in the anaerobic cell-free system, rat liver was homogenized in a sucrose medium (0.35 M sucrose, 0.035 M KHCO$_3$, 0.025 M KCl, and 0.004 M MgCl$_2$) under the conditions described previously (2). The homogenate was centrifuged for 10 minutes at 15,000 × g, and the supernatant fluid containing microsomes and the soluble cell fraction was used without further fractionation.

Materials—P^{32} was obtained from the Oak Ridge National Laboratories.

PP³² was prepared by pyrolysis of P³² and contained less than 17 per cent of P³². (The pH 5 enzyme does not catalyze the P-ATP exchange.)

ATP, CTP, and UTP were obtained from the Pabst Laboratories. The Sigma Chemical Company supplied crystalline ATP, ITP, GDP, and GTP. Paper electrophoresis of this sample of GTP indicated the presence of considerable GDP.

Hydroxylamine was prepared salt-free from the hydrochloride by the method of Beinert et al. (5) and stored frozen.

Amino acids used in these studies were usually products from the Nutritional Biochemicals Corporation or the Schwarz Laboratories, and the purity of all had been checked by paper chromatography. Dr. Jesse Greenstein's group kindly supplied pure L-alanine, L-serine, L-isoleucine, and L-valine. The following twelve amino acids were used throughout: leucine, isoleucine, valine, glycine, threonine, histidine, phenylalanine, tryptophan, serine, alanine, arginine, and lysine. Glutamic and aspartic acids were omitted because of the activation of other than α-carboxyl groups, proline because of its possible conversion to glutamic acid, cysteine because of its complicating sulfhydryl effect, tyrosine because of its low solubility, and methionine for reasons which become apparent below.

The hydroxamic acids of leucine, glycine, alanine, lysine, valine, and isoleucine were generously supplied by J. D. Gregory and S. Genuth. Methionine hydroxamic acid was prepared by the method of Safir and Williams (6) from methionine isopropyl ester, a gift of Dr. Max Brenner. Since paper chromatography or paper electrophoresis of these amino hydroxamic acids showed the presence of small amounts of impurities, particularly the corresponding amino acid, they are being purified further to provide exact standards.

Exchange Studies—The pH 5 enzyme was incubated for 8 minutes at 37° in a volume of 1.0 ml. with 100 μmoles of Tris buffer, pH 7.6, 5 μmoles of ATP, 1 μmole of PP³², pH 7.5, containing 100,000 to 200,000 c.p.m., 2 μmoles of $MgCl_2$, and amino acids as indicated. The low Mg^{++}:ATP ratio prevents enzymatic PP hydrolysis during the incubation. The reaction was stopped by adding 0.3 ml. of 25 per cent trichloroacetic acid, and the ATP and PP were separated and determined by charcoal adsorption according to the method of Crane and Lipmann (7). Under these conditions neither ATP nor PP was hydrolyzed. The results are expressed arbitrarily as per cent exchange, which is calculated as follows:

$$\frac{\text{C.p.m. per } \mu\text{mole ATP}}{\text{Total c.p.m. per } \mu\text{mole (ATP + PP)}} \times 100$$

This gives the specific activity of the ATP as per cent of the value which would be obtained at equilibrium.

Hydroxamic Acid Formation—The pH 5 enzyme was incubated at 37°

for 50 minutes in a final volume of 2.0 ml. with 20.0 μmoles of the mono-magnesium, dipotassium salt of ATP (except in experiments in which the Mg:ATP ratio was varied), about 2.4 mmoles of salt-free hydroxylamine, and amino acids according to the plan of the experiment. Hydroxamic acid was measured directly on a 1.0 ml. aliquot of the reaction mixture by adding 3 ml. of a solution of 10 per cent $FeCl_3$, 5 per cent trichloroacetic acid, and $\frac{2}{3}$ N HCl, with use, as an internal standard, of an equimolar mixture of the first six amino hydroxamic acids mentioned above, or when appropriate, the specific amino hydroxamic acid. At the final pH of the $FeCl_3$ color development (about 1.0), the amino hydroxamic acids so far tested vary in color yield by no more than 20 per cent, most of them by less than that. After inactivating the enzyme in the remaining 1.0 ml. of incubation medium by placing the tubes in boiling water for 2 minutes, inorganic orthophosphate was determined by the method of Fiske and Subbarow on a 0.1 ml. aliquot, and was compared to a zero time control. PP was determined on a similar aliquot after ATP had been adsorbed on charcoal by the method of Flynn et al. (8).

Paper Chromatography of Reaction Products—Secondary butanol-formic acid-water in the ratio 75:15:10 and Whatman No. 3 filter paper were used for demonstrating amino hydroxamic acids. Genuth and Gregory[2] had previously found this system to give good separation of these substances. Descending chromatograms were run for 12 to 18 hours and then sprayed with acidic $FeCl_3$ solution. Internal standards of known amino hydroxamic acids were used. The samples were prepared for chromatography by placing the incubation mixture in boiling water for 2 minutes, removing the protein by centrifugation, evaporating the supernatant solution to dryness, and desiccating for 24 hours *in vacuo* over concentrated H_2SO_4 to remove NH_2OH. The residue was redissolved in water and placed on paper.

Results

General Characteristics of Amino Hydroxamic Acid-Forming System—The activity of the pH 5 enzyme varied from day to day but usually corresponded to 0.4 μmole of hydroxamic acid formed per mg. of protein per hour at 37°. The test was made with 10 μmoles of MgK_2ATP, 1.2 mmoles of NH_2OH (pH 7.5), and 2 μmoles each of twelve amino acids, in a final volume of 1 ml. The pH 5 enzyme is about 5 times as active as the original soluble protein fraction. There was linear increase in hydroxamic acid formed with increasing Mg ATP concentrations up to 7 μmoles per ml., with maximal response at 10 μmoles. Fig. 1 shows the accumulation of hydroxamic acid with time and indicates that the reaction proceeds

[2] Genuth, S., and Gregory, J. D., personal communication.

linearly for about 50 minutes.   Fig. 2 relates hydroxamic acid appearance and NH_2OH concentration.

Although the enzymatic activity of the original soluble protein fraction of rat liver is rapidly lost at $-10°$, the pH 5 enzyme can be stored frozen with little loss in activity.   All enzymatic activity is lost upon heating at $50°$ for 3 minutes.   The stability properties of the system apply to the PP-ATP exchange reaction as well.   The base-line exchange and hydrox-
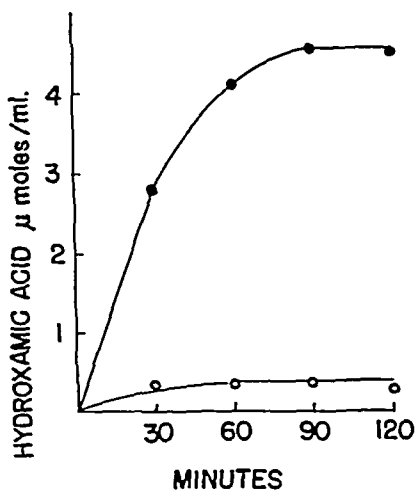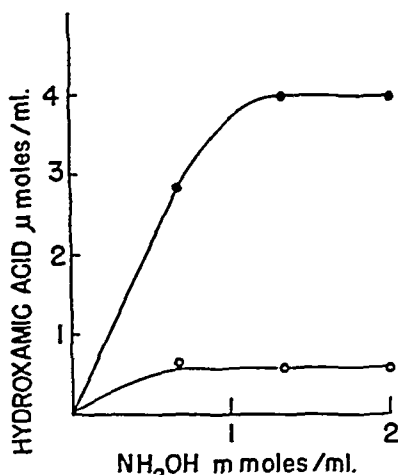

FIG. 1. Time curve for hydroxamic acid formation.   7.8 mg. of pH 5 enzyme were incubated with 1.2 mmoles of NH_2OH, 10 μmoles of MgK_2 ATP, and 2 μmoles of each of twelve amino acids in a volume of 1 ml. at $37°$.   O, without amino acids; ●, with amino acids.

FIG. 2. Relation between hydroxamic acid formation and hydroxylamine concentration.   11.6 mg. of pH 5 enzyme were incubated at $37°$ for 60 minutes with 10 μmoles of MgK_2 ATP, 2 μmoles of each of twelve amino acids, and varying amounts of NH_2OH, in a volume of 1 ml.   O, without amino acids; ●, with amino acids.

amic acid formation are less than one-tenth of the values obtained in the presence of amino acids (see Figs. 1 and 2).

Stoichiometry of Pyrophosphate and Amino Hydroxamic Acid Formation— It is possible to show good stoichiometry for hydroxamic acid formed, labile phosphate lost, and orthophosphate or pyrophosphate accumulated. Because of the high pyrophosphatase activity of the preparation, at optimal magnesium concentrations all pyrophosphate formed is converted to orthophosphate, and therefore 2 equivalents of phosphate are formed for every mole of hydroxamic acid formed.   However, by adding fluoride or, as is illustrated in Fig. 3, reducing the Mg^{++} concentration, pyrophosphatase can be completely inhibited, while the activation enzymes retain

some activity. Under these conditions, pyrophosphate accumulates in an amount equal to the amount of hydroxamic acid formed. At higher Mg^{++}:ATP ratios, it can be seen that the orthophosphate divided by 2 plus the pyrophosphate equals the hydroxamic acid formed. Thus it was established that pyrophosphate is indeed the product of the reaction.

Reaction in Absence of Acceptor—*There is no measurable accumulation of phosphate due to amino acids in the absence of hydroxylamine.* It is worth
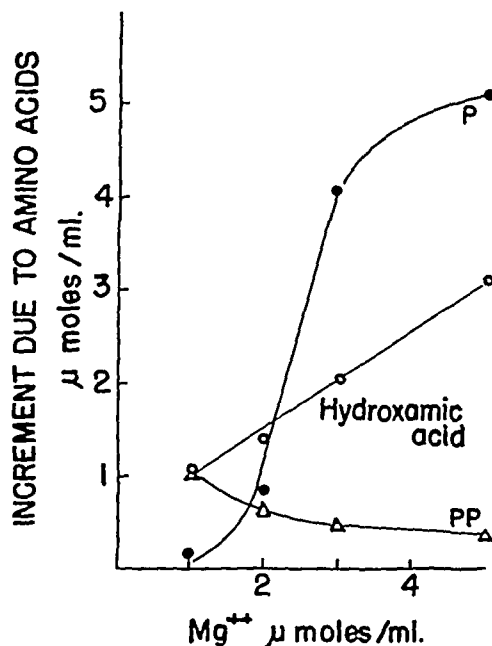

FIG. 3. Orthophosphate, pyrophosphate, and hydroxamic acid formation as a function of Mg^{++}:ATP ratio. 2 μmoles of each of twelve amino acids, 1.2 mmoles of NH_2OH, and 7 mg. of pH 5 enzyme in 1 ml. were incubated for 60 minutes. ATP was held constant at 10 μmoles and $MgCl_2$ was varied as indicated. (Other experiments reveal that with Mg^{++}:ATP below 0.1 the PP and hydroxamic acid are equal and decrease with decreasing Mg^{++}:ATP.) These data are from one of three similar experiments.

restatement at this point that AMP inhibits neither the exchange nor the hydroxamic acid formation and, more important, that C^{14}-labeled AMP fails to exchange with ATP under conditions in which PP^{32}-ATP exchange is vigorous. Furthermore, as would be expected from equilibrium considerations, NH_2OH markedly inhibits PP^{32}-ATP exchange.

Although the above evidence indicates that the activated amino acid and AMP are bound to the enzyme in the absence of an acceptor, some attempt has been made to detect a trace of free AMP $\sim$ amino acid compound. Five C^{14}-labeled amino acids (leucine, glycine, valine, isoleucine, phenylalanine) were incubated with ATP and the enzyme preparation. The products of the reaction, after deproteinization, were then adsorbed on

charcoal at pH 4.0. After six washings with buffer and inert amino acids, the charcoal was boiled for 60 minutes with 6 N HCl. These conditions would split the ribose from AMP and thus release any bound C^{14}-amino acids into the supernatant solution. There was, however, no greater radioactivity released than in controls in which the enzyme was heated, Mg was omitted, or adenosine diphosphate replaced ATP.

Separate Activation of Naturally Occurring L-Amino Acids—Both PP-ATP exchange and hydroxamic acid formation are dependent upon L-amino acids. The D isomers are inactive and do not inhibit the activation of the L isomers. The extent of hydroxamic acid formation and exchange produced by a number of amino acids are reported in Table I. It can be seen

TABLE I

Hydroxamic Acid Formation and PP-ATP Exchange with Amino Acids

Amino acid, 5 μmoles each	Per cent exchange	Hydroxamic acid formed
		μmoles
Tryptophan............	2.4	1.1
Leucine....................................	14.7	1.0
Alanine	1.2	0.7
Lysine..	0.2	0.3
Valine	5.1	0.1
All 5. .	17.3	3.0

Exchange was measured by incubating 4 mg. of pH 5 enzyme with 5 μmoles of ATP, 1 μmole of Mg^{++}, and 4 μmoles of PP (containing 280,000 c.p.m.) in 1 ml. for 8 minutes at 37°. Hydroxamic acid formation was determined after incubating 9.7 mg. of protein with 10 μmoles of Mg ATP, 1.2 mmoles of NH_2OH, and the amino acids indicated in a volume of 1 ml. for 50 minutes at 37°.

that in both reactions the effect of individual amino acids is additive. Indeed, even at high concentrations, there is no evidence of competition between amino acids for activation sites. Two further points to be noted are (1) the great difference in activity for the various amino acids and (2) the fact that there is no parallelism between hydroxamic acid formation and exchange.

Methionine-Activating Enzyme—During earlier attempts to fractionate the soluble protein fraction with ammonium sulfate, a peak of activity for hydroxamic acid formation with the group of twelve amino acids was found between 35 and 45 per cent saturation. However, when the amino acids were tested individually, methionine accounted for essentially all of the activity. Table II shows that this ammonium sulfate fraction catalyzes the conversion of methionine to methionine hydroxamic acid with

concomitant production of approximately 2 orthophosphate equivalents. It should be noted that at the lower Mg^{++} concentration pyrophosphatase is inhibited and pyrophosphate accumulates as the product. This fraction

TABLE II

Methionine Carboxyl Activation by Ammonium Sulfate Fraction from Rat Liver Soluble Protein Fraction

	Products formed			
	Hydroxamic acid	P	PP	P/2 + PP
	μmoles	*μmoles*	*μmole*	*μmoles*
10 μmoles Mg^{++}				
No methionine	0.1	3.8	0.1	
5 μmoles methionine	2.1	6.5	0.3	
Δ	2.0	2.7	0.2	1.6
4 μmoles Mg^{++}				
No methionine	0.2	2.0	0	
5 μmoles methionine	1.7	3.4	0.7	
Δ	1.5	1.4	0.7	1.4

3.5 mg. of protein were incubated at 37° for 50 minutes with 10 μmoles of ATP, 10 or 4 μmoles of Mg^{++}, and 1.2 mmoles of NH$_2$OH. Methionine hydroxamic acid was used as standard.

TABLE III

Nucleotide Specificity of Activation System

Nucleotide added	Hydroxamic acid formed
	μmole
ATP 2.5 μmoles	0.79
CTP 2.0 "	0.1
UTP 2.5 "	<0.1
ITP 2.5 "	<0.1
GTP 2.5 "	<0.1
None	0

4.6 mg. of the pH 5 enzyme were incubated at 37° for 60 minutes with nucleotides, 1.2 mmoles of NH$_2$OH, and 24 μmoles of twelve amino acids.

also catalyzes a methionine-dependent PP^{32}-ATP exchange. The methionine hydroxamic acid has been identified by paper chromatography.

Nucleotide Specificity—Further progress in delineating the components of the amino acid incorporation system (9) has revealed that the washed pH 5 enzyme, when combined with crystalline ATP and microsomes that have been centrifuged out of a diluted 15,000 × *g* supernatant solution,

no longer catalyzes incorporation of amino acids into protein. If the system is supplemented with GDP or GTP, however, activity is restored. The nucleotide is ineffective in the absence of ATP, and other nucleotides will not replace the guanosine derivatives. Thus it was of great interest
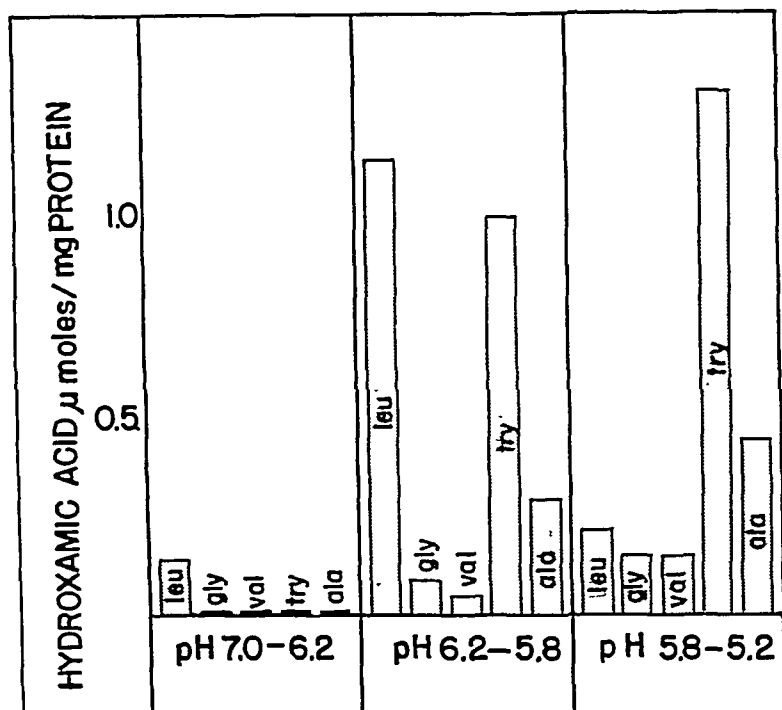

FIG. 4. Activity of three isoelectrically precipitable protein fractions toward five amino acids. 5 μmoles of each amino acid were incubated with from 3 to 7 mg. of protein of each fraction. The fractions were prepared by adding 0.1 N HCl slowly with vigorous stirring to rat liver supernatant fraction containing about 20 mg. of protein per ml. until the desired pH (by glass electrode) was reached. The precipitate was collected at 15,000 × *g* and redissolved in 0.1 M Tris buffer, pH 7.6. All operations were performed as rapidly as possible at 0°. A number of other isoelectric fractions of this kind produced similar distribution of activity.

to determine whether GDP or GTP is required for amino acid activation. The washed pH 5 enzyme was found to be fully active in the amino acid-dependent PP-ATP exchange with crystalline ATP and twelve L-amino acids, and GDP was found to have no effect on the exchange. In addition GTP cannot replace ATP as the source of energy for activation of the group of twelve amino acids tried (Table III).

Further Fractionation of pH 5 Enzyme—To determine whether separate enzymes mediate the activation of individual amino acids, several iso-

electrically precipitable fractions were tested for their activity toward a representative group of amino acids. Fig. 4 is typical of a number of such experiments. It can be seen that there is indeed a clear-cut difference in activity of the fractions toward the amino acids tested. There would appear to be separation of activity for leucine in the fractions obtained at pH 6.2 to 5.8. This would indicate that a separate protein mediates the activation of leucine. In another experiment, activity for alanine was also separated by isoelectric precipitation. With use of two of these

TABLE IV

Hydroxylamine Inhibition of Incorporation of L-Leucine-C[14] into Protein

The figures are in counts per minute per mg. of washed protein determined as described previously (1).

Hydroxylamine	ATP precursors		
	Phosphoenolpyruvate, 10 μmoles per ml.	Phosphocreatine, 20 μmoles per ml.	Carbamyl phosphate[*] 10 μmoles per ml., and kinase[†]
mmoles per ml.			
0	79	88	87
0.05	70		57
0.2	48	35	36
0.6	27	22	22
1.2	15	14	11

Each flask contained, in a final volume of 1.0 ml., 0.1 μmole of L-leucine-1-C[14] 82,000 c.p.m. (supplied by Dr. R. B. Loftfield), 0.7 ml. of supernatant fluid (with 15.5 mg. of protein) centrifuged at 15,000 × g from a rat liver homogenate prepared as described under "Experimental." The incubation time was 10 minutes at 37° under 5 per cent CO_2-95 per cent N_2.

* Kindly provided by Dr. L. B. Spector.
† A crude *Streptococcus faecalis* R extract, 0.2 mg. of protein per flask, supplied the necessary carbamate kinase. The extract was a gift from Dr. M. E. Jones.

fractions, one active for leucine and the other for alanine, it has been possible to demonstrate the appearance on paper chromatograms of $FeCl_3$-reacting spots indistinguishable from known leucine and alanine hydroxamic acids respectively. Preliminary studies on heat inactivation of the pH 5 enzyme also point to separate activating enzymes.

Hydroxylamine Inhibition of Incorporation of C[14]-Amino Acid into Protein—The fact that the pH 5 enzyme preparation which produces amino acid activation by forming enzyme-bound amino acyl ∼ AMP complexes is also required for the incorporation of C[14]-amino acids into protein in the cell-free system from liver suggests that this activation may be the first step in the incorporation reaction. Another suggestive piece of evidence is that the incorporation is inhibited by hydroxylamine (Table

IV) and that the inhibition roughly parallels the amino hydroxamic acid formation with increasing concentration of hydroxylamine. Table IV demonstrates that the inhibition is the same regardless of which of three different ATP generating systems is used.

DISCUSSION

The data presented in the foregoing support a hypothesis for amino acid activation which is schematically presented in Fig. 5. The R group of the amino acids and the adenine moiety of ATP would both be bound
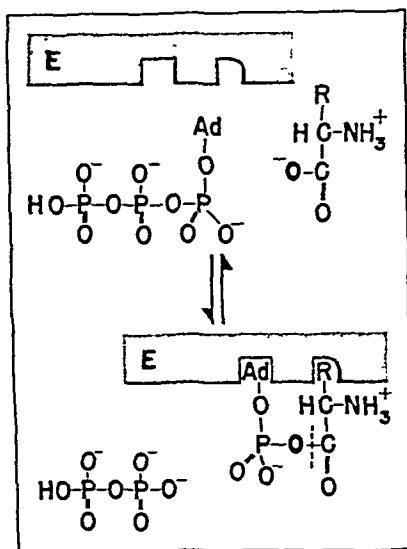

FIG. 5. Schematic representation of amino acid carboxyl activation by ATP and the pH 5 enzyme. Ad = adenosine. O indicates the attacking carboxyl oxygen which would remain with the nucleotide moiety upon subsequent splitting of the activated compound (dash line).

by the specific activating enzyme. This binding would create favorable conditions for a cleavage of ATP by an attack of the carboxyl oxygen upon the stable phosphate of ATP and ejection of the pyrophosphate. The bond energy would thus be retained in a carboxyl-phosphate linkage. The result would be an enzyme-bound, carboxyl-activated, amino acid $\sim$ AMP compound. Magnesium may participate in the labilizing of the AMP $\sim$ pyrophosphoryl linkage. Each activating enzyme or site appears to act independently, forming an AMP compound with a specific amino acid, without interference from other amino acids.

The enzyme-bound amino acid $\sim$ AMP compound would then react with a natural cellular acceptor: either another nucleotide carrier or the nucleic acid of the microsome. The next step would be polypeptide

condensation, which appears to occur in the ribonucleoprotein particles of the microsome fraction (10). In the present experiments the artificial trap, hydroxylamine, permits the removal of the activated amino acid and regeneration of the enzyme for recycling. In any case, the point of cleavage of the carboxyl-phosphate linkage by the amine acceptor would leave the carboxyl oxygen on the AMP.

The PP-ATP exchange which depends upon amino acid, the failure of AMP to exchange with ATP, the lack of product accumulation in the absence of hydroxylamine, the appearance of amino hydroxamic acids and pyrophosphate in the presence of hydroxylamine, and the evidence suggesting separate activating enzymes are all consistent with this scheme.

A number of recent studies suggest that this mechanism of activation may be of quite general character when synthetic reactions involve a pyrophosphate split of ATP. Maas (11) has good evidence that the activation of pantoate for synthesis of pantothenate proceeds by an identical mechanism. Berg (12) has shown that the activation of acetate involves the initial formation of an acetyl $\sim$ AMP compound. Evidence for the existence of an AMP $\sim$ CO_2 compound has been presented (13). Hilz (14) has observed the possible occurrence of an AMP $\sim$ sulfate compound in a system in which ATP supplies the energy for sulfate activation. The ATP-dependent synthesis of benzoyl coenzyme A in hippuric acid synthesis seems to involve a pyrophosphate split of ATP (15). Finally, an amino acid-dependent PP-ATP exchange has been found in a variety of microorganisms (16). The present studies support the suggestion made by Lipmann 15 years ago (17) that the phosphate bond energy of ATP might be used for carboxyl activation of amino acids.

The extent of dissociation of the acyl $\sim$ AMP compound from the specific enzyme seems to vary considerably in the different systems described above. It appears that, at least in the present system, dissociation of the AMP $\sim$ amino acid compound from the enzyme is of such small extent that its free existence has thus far not been demonstrated. The interesting observation (Table I) that there is no parallelism between hydroxamic acid formed and PP-ATP exchange suggests that the amino acids differ not only in the rate at which they are activated (as measured by rate of exchange) but also in the extent to which the activation site is accessible to hydroxylamine (as measured by hydroxamic acid formation).

The stability properties and fractionation characteristics of this amino acid activation system closely parallel those of the soluble enzyme preparation required for incorporation of labeled amino acids into microsome protein. The inhibition of incorporation by hydroxylamine is also suggestive.

The rate of incorporation of leucine into whole liver protein *in vivo* has

been estimated as at least 5 μmoles per gm. of protein per hour (3). In 1 gm. of liver protein there are about 400 mg. of soluble protein fraction which can form about 13 μmoles of leucine hydroxamic acid per hour. This compares favorably with the figure for incorporation *in vivo*.

The fact that the activation of the amino acids so far tested is specific for ATP and is uninfluenced by GTP suggests that the requirement for the latter nucleotide in the incorporation of amino acids into protein is at a stage following amino acid activation.

It is of interest that methionine can be activated at the carboxyl group by an ammonium sulfate-precipitable enzyme. Since this fraction is similar to one described by Cantoni (18) which contains the enzymes that synthesize adenosylmethionine from ATP and methionine, it is conceivable that carboxyl activation of methionine might be an intermediate stage in adenosylmethionine synthesis.

SUMMARY

An enzyme preparation obtained from the soluble protein fraction of rat liver by precipitation at pH 5.2 to 5.1 has the following properties.

1. It catalyzes an exchange of PP^{32} with ATP which is essentially L-amino acid-dependent. The exchange is dependent both on the concentration and the number of amino acids added.

2. When incubated with ATP, a mixture of L-amino acids, and a high concentration of hydroxylamine, hydroxamic acid is produced. The hydroxamic acids of leucine and alanine have been identified by paper chromatography. At the optimal Mg^{++} concentration, 2 moles of orthophosphate are formed per mole of hydroxamic acid. At low Mg^{++} concentrations, 1 mole of pyrophosphate is formed per mole of hydroxamic acid, and no orthophosphate appears. No products appear in the absence of hydroxylamine.

3. In all cases D-amino acids are inert.

4. Hydroxylamine inhibits PP-ATP exchange.

5. Preliminary fractionation and heat inactivation studies suggest that separate enzymes are involved in the activation of several amino acids.

Incubation of a 35 to 45 per cent ammonium sulfate fraction of the soluble protein fraction of rat liver with methionine, ATP, and a high concentration of hydroxylamine leads to the formation of methionine hydroxamic acid. Other amino acids are not activated by this or other ammonium sulfate fractions.

The relation of these reactions to the activation of amino acids for protein synthesis is discussed.

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THE PREPARATION ON A MICROGRAM SCALE OF SOME DERIVATIVES OF ALDOSTERONE: THEIR USE IN PAPER CHROMATOGRAPHY

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In order to identify a substance by means of paper chromatography it is highly desirable to compare the running rates of as many of its derivatives as possible with the running rates of comparable derivatives of known substances. When only microgram quantities are available, the preparation of derivatives in good yield often presents a problem. It is the primary purpose of this communication to describe the preparation of three derivatives of aldosterone on a microgram scale and their use to establish the identity of two preparations of crystalline aldosterone and the identity of aldosterone isolated from the "amorphous fraction." Some bioassay data are also included.

Aldosterone (147 γ) was acetylated to give 50 γ of crystalline 21-acetate, m.p. 193–195°. Simpson and associates (1) reported the melting point as 198–199°; Ham and associates (2) gave 193–196.5°. Another monoacetate, m.p. 217–219°, was described in previous publications (3, 4). This acetate now must be regarded as the 18-acetate of the 11,18-lactol structure of aldosterone.

The 18,21-diacetate, prepared from 1.6 mg. of aldosterone, could be obtained only in the form of discrete pellets in which a crystalline structure could not be discerned. They melted at about 80°. Simpson and associates (1) gave a melting point of about 70° for the diacetate. These preparations were used for comparison with corresponding preparations derived by similar means from a few micrograms of aldosterone.

In a bioassay the activity of the 21-acetate could not be distinguished from that of aldosterone (Fig. 1). Similar results were reported by Ham and associates (2); Simpson and associates (1) found 1 γ of aldosterone equivalent to 1.25 γ of aldosterone 21-acetate. Preliminary assays (3, 4) of the 18-monoacetate indicated an activity about 25 times that of deoxycorticosterone acetate, but additional assays showed that that factor should be reduced to about 15. The activity of the 18,21-diacetate proved to be

about 0.01 that of aldosterone or about equal to that of deoxycorticosterone acetate.

Aldosterone 21-acetate was oxidized to a neutral substance regarded as aldosterone-11,18-lactone 21-acetate (5). Bioassay of this substance showed no sodium-retaining activity at a level of 0.50γ per rat.

One of the preparations of aldosterone (81γ) was kindly furnished by Professor Reichstein for direct comparison with the preparation of Mattox and associates (3, 4). This material was divided into five parts. One part was assayed in adrenalectomized rats as previously described. It proved
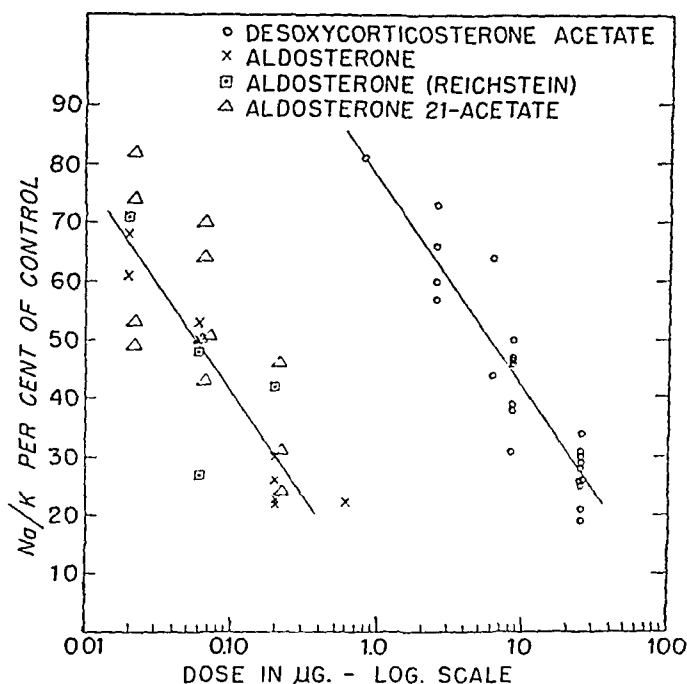

FIG. 1. Assay of adrenal cortical hormones in adrenalectomized rats. Each point represents results obtained with five animals.

to have the same activity as our substance within the limits of the assay procedure; that is, approximately 100 times the activity of deoxycorticosterone acetate. The assay values are plotted in Fig. 1.

Equal amounts of our substance and that from Reichstein were chromatographed on paper in both the toluene-propylene glycol and benzene-formamide systems of Zaffaroni and Burton (6). Cortisone was included for comparison. The 21-monoacetates and the diacetates were prepared and chromatographed in formamide with 1:1 cyclohexane-benzene. 11-Dehydrocorticosterone and its acetate were included in the respective chromatograms. It is evident from Fig. 2 that the two samples moved at the same rate in toluene-propylene glycol and at the same rate in benzeneformamide, although the mobility with respect to cortisone was different

in the two solvent systems.   The monoacetates of the two samples moved at identical rates, and also the diacetates of the two samples had identical mobilities.   Presumably the small areas near the origin in the chromatography of aldosterone 21-acetate are due to unacetylated aldosterone. These results are considered to establish firmly the identity of the two preparations.
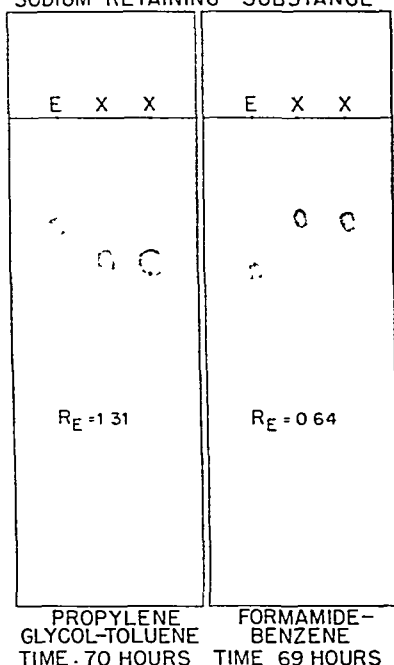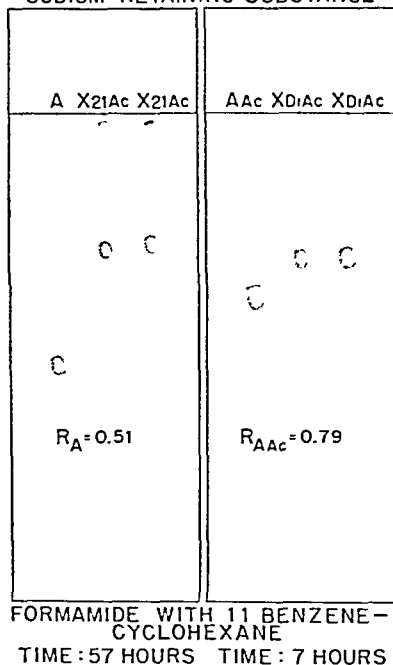
FIG. 2. Chromatography of adrenal hormones and derivatives. Each sample represents 19 γ of steroid. E = cortisone, X = aldosterone, A = 11-dehydrocorticosterone, Ac = acetate. R_E = 1.31 indicates that X moves 1.31 times as fast as E. In each chromatogram our sodium-retaining compound is shown in the right column.

An amorphous fraction (7) of an extract of 630 pounds of adrenal glands was prepared by the laborious procedure of successive distributions between benzene and water and crystallizations.   Since most of the activity was lost during the preparation, the amount of aldosterone isolated from this amorphous fraction has little significance.   The amorphous fraction was chromatographed several times and an amorphous product was isolated.   It was identified as aldosterone by bioassay as the free substance and as the 21-acetate, by its chromatographic mobility in two solvent systems, and by the chromatographic mobilities of its 21-acetate and its 18,21-diacetate.

EXPERIMENTAL

√ *Chromatographic Procedures*—Filter paper (Whatman No. 1, "special for chromatography") was washed by immersion in 95 per cent alcohol for a week with a change of solvent every 2 days. It was then rinsed twice with benzene and allowed to dry. For impregnation with the stationary phase, the paper was drawn through a freshly prepared 1:1 mixture of methanol and the stationary phase (propylene glycol or formamide), then hung from a glass rod, and allowed to dry for 1.5 hours. At this time the steroids were applied along a line 10 cm. from the narrow edge of the paper, with a 2.5 to 3.0 cm. space separating the individual compounds or fractions. At the end of 2 hours from the time the paper was impregnated, it contained about 49 per cent of its weight of stationary phase. At this time it was placed in a chromatography jar, and the chromatogram was developed with the mobile phase which had been saturated with the stationary phase. When the solvent front was to flow off the paper, the bottom edge of the paper was pinked to give pointed edges at 2 cm. intervals.

Preparation of Aldosterone 21-Acetate—11,20-Diketopregnane-3 α,21-diol was used as a model to study the rate of 21-acetylation, and the conditions which gave the best yield of 21-acetate were then applied to aldosterone. To 147 γ of crystalline aldosterone in 1 ml. of glacial acetic acid were added 1.2 ml. of a mixture of 1 ml. of acetic anhydride and 5 ml. of 3 M pyridine in glacial acetic acid. After 1.25 hours at room temperature, 10 ml. of chloroform were added, then ice, and 3 ml. of N hydrochloric acid. The mixture was shaken thoroughly, and the chloroform was separated and washed with water, twice with a dilute solution of sodium bicarbonate, and twice with water. After filtration through anhydrous sodium sulfate (previously washed with chloroform) in a sintered glass funnel, the chloroform was evaporated in a stream of carbon dioxide while warming in a water bath. After addition and removal of a small portion of methanol, the residue was moistened with a small drop of acetone, and 0.2 ml. of dry ether was added. Crystals formed; after refrigeration the solvent was removed with a capillary pipette, and the crystals were washed with a few drops of dry ether. The crystals were dissolved in a small drop of acetone. Addition of 3 drops· of ligroin and refrigeration gave crystals (50 γ) which melted at 193–195° (Fisher-Johns). The same conditions as those just described were applied to the smaller amounts available from Professor Reichstein and from the amorphous fraction.

When chromatographed on paper in formamide with 1:1 cyclohexane-benzene, the 21-acetate moved 0.082 times as fast as aldosterone diacetate and at 0.51 times the rate of 11-dehydrocorticosterone. In formamide-1:1 benzene-cyclohexane, aldosterone 21-acetate moved 0.59 times as fast as aldosterone-11,18-lactol 18-acetate, m.p. 217–219°.

Aldosterone Diacetate—Aldosterone (1.6 mg.) was treated with 4 drops of acetic anhydride and 4 drops of pyridine. After 20 hours the pyridine and acetic anhydride were removed in a stream of carbon dioxide. Methanol was added and removed twice. After unsuccessful attempts to crystallize the material from dry ether and ether-petroleum ether, it was dissolved in 1.5 ml. of hot petroleum ether. On cooling, small solid balls separated on the walls of the tube; no crystalline structure could be seen under a microscope. The solid melted gradually, becoming liquid at about 80°.

Oxidation of Aldosterone 21-Acetate to 11,18-Lactone 21-Acetate—A solution of 59 γ of aldosterone 21-acetate in 1 drop of acetic acid was cooled to 13° and treated with 1 drop of a solution of 1 gm. of chromium trioxide in 1 ml. of water and 99 ml. of glacial acetic acid, also cooled to 13°. After 10 minutes at this temperature, 0.5 ml. of water and 5 ml. of ethyl acetate were added. The ethyl acetate was separated, washed twice with 0.5 ml. of water, and evaporated to dryness in a stream of carbon dioxide. A blank was carried through this procedure which had been shown to convert corticosterone acetate to 11-dehydrocorticosterone acetate in good yield. The products of the oxidation were transferred with the aid of 1:1 chloroform-methanol to paper impregnated with formamide, and the chromatogram was developed for 22 hours with 1:1 cyclohexane-benzene saturated with formamide. The lactone moved 2.2 times as fast as aldosterone 21-acetate. The lactone from the 59 γ of aldosterone 21-acetate was eluted with alcohol. The alcohol was removed and the residue dissolved in 50 ml. of chloroform. Formamide was washed out with three 5 ml. portions of water, the chloroform was removed, and the ultraviolet spectrum determined in 5.0 ml. of methanol. The optical density at 238 mμ, corrected for the absorption of the blank, indicated the presence of 49.6 γ of lactone.

Aldosterone from Amorphous Fraction—An aqueous solution of the amorphous fraction was taken to dryness and chromatographed on a 1.8 × 50 cm. column which contained 60 gm. of 100 mesh silica gel (Davison) impregnated with 30 ml. of propylene glycol; propylene glycol-saturated toluene was used as the mobile phase. Fractions of eluate were collected every 12 hours, the rate of flow being about 10 ml. per hour. Fraction 17 contained the peak concentration of cortisone. Fractions 14 to 19 (which represented the eluate of 1476 to 2138 ml.) were combined, and the solvent was removed. One-half of the residue was chromatographed on paper (35 cm. wide) in formamide-benzene for 96 hours. The area corresponding in mobility to aldosterone was eluted and chromatographed in the same system on a 3 × 56 cm. strip of paper. The main band moved 0.73 times as fast as cortisone. Bioassay indicated the presence of 57 γ of aldosterone. Chromatography on paper in propylene glycol-toluene for 95 hours gave an ultraviolet-absorbing zone which had moved at the same rate as aldosterone.

The material was recovered and converted to the 21-acetate, as previously described. The 21-acetate was chromatographed on paper in formamide with 1:1 cyclohexane-benzene for 64 hours. The zone at 12.4 to 15.5 cm. from the origin had moved at the same rate as aldosterone 21-acetate and 0.52 times as fast as 11-dehydrocorticosterone. The extinction of the eluted sample, corrected with an appropriate blank, indicated the presence of 42 γ of aldosterone 21-acetate. In the bioassay the compound was approximately 100 times as active as deoxycorticosterone acetate and therefore as active as aldosterone. The diacetate of the material from the "amorphous fraction" was prepared as described for crystalline aldosterone and chromatographed on paper with authentic aldosterone diacetate for comparison. The procedure was the same as for aldosterone 21-acetate except that the time of development was only 6 hours. The running rate of the diacetate from the amorphous fraction was identical with that of aldosterone diacetate and was 0.82 times that of 11-dehydrocorticosterone acetate.

SUMMARY

Small scale preparation of aldosterone 21-acetate, 18,21-diacetate, and 11,18-lactone 21-acetate and their use in the paper chromatographic identification of aldosterone have been described. With the aid of these derivatives as well as by comparative bioassays, the identity of the preparations of Simpson and associates and of Mattox and associates was demonstrated.

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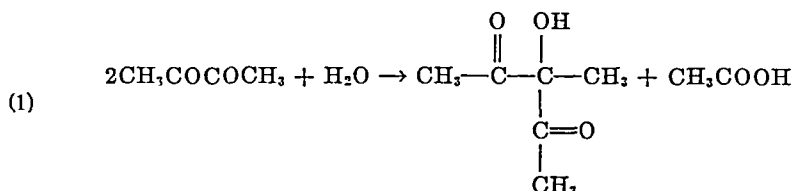
ACYLOIN CONDENSATION REACTIONS OF PYRUVIC OXIDASE*

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Almost all enzymes capable of decarboxylating pyruvate have been shown to catalyze DPT[1]-dependent condensation reactions yielding acyloins such as AMC (1–7). Recent work of Schweet and coworkers (8) has demonstrated that in animal tissues these condensations are due to the activity of pyruvic oxidase. Purified preparations of pyruvic oxidase from pigeon breast muscle have the same ratio of acyloin formed to pyruvate oxidized as do crude homogenates. In preparations of α-carboxylase from wheat germ there is a constant ratio of decarboxylation to AMC synthesis throughout a 2700-fold purification (5), and the different activities have identical requirements for DPT and a metallic ion.

Preparations of animal pyruvic oxidase can utilize diacetyl as an analogue of pyruvate. Schweet and coworkers (8) have shown pyruvic oxidase to be identical with the diacetyl mutase first described by Green and coworkers (9). Studies in this laboratory have revealed a new pathway for the bacterial dissimilation of diacetyl (10). In this system diacetyl is quantitatively converted to acetic acid and diacetylmethylcarbinol (Equation 1) in a reaction similar to that involving the formation of α-acetolactate from pyruvate by AMC-producing bacteria (Equation 2).

$$(1) \quad 2CH_3COCOCH_3 + H_2O \rightarrow CH_3-\overset{\overset{O}{\|}}{C}-\overset{\overset{OH}{|}}{\underset{\underset{CH_3}{|}}{\underset{\underset{|}{C=O}}{C}}}-CH_3 + CH_3COOH$$

This finding led to a reinvestigation of the condensation reactions in animal tissue. DAMC, in addition to AMC, was found to be produced from diacetyl by both animal and bacterial pyruvic oxidase preparations. When pyruvate was the substrate, both AMC and α-acetolactate were the condensation products formed. With diacetyl or pyruvate as substrate,

[1] The following abbreviations are used: DPT, diphosphothiamine; AMC, acetylmethylcarbinol (acetoin); DAMC, diacetylmethylcarbinol.

AMC formation occurred as a consequence of free acetaldehyde production by these enzyme preparations.

Methods and Materials

The pigeon breast muscle preparations were made according to Jagannathan and Schweet (11). In most experiments relatively crude fractions (Fractions 1 and 2) were used. The pig heart carboxylase was prepared by the method of Green and coworkers (4). *Escherichia coli* (Crookes strain) was grown in the dismutation medium previously described (12) and the cells were harvested with a Sharples centrifuge after 19 to 22 hours of growth at 30° with forced aeration. The cells were resuspended in twice their volume of water and subjected to sonic vibration for 40 minutes with a Raytheon 10 kc. oscillator. The sonicate was then centrifuged at 25,000 × g for 30 minutes and the precipitate discarded. α-Acetolactic decarboxylase was prepared from extracts of *Aerobacter aerogenes* (12).

In tests for condensation products other than α-acetolactate, reaction mixtures were generally deproteinized by placement of the contents of a reaction flask, together with several washings of the same flask, in a centrifuge tube containing 0.5 ml. of 10 per cent trichloroacetic acid. The precipitated protein was removed by centrifugation and the supernatant fluids made to volume, usually 10 ml. Zinc hydroxide precipitation (13) was used when α-acetolactate was present. For determination of volatile acid as well as condensation products, 5 per cent sulfuric acid was used in place of trichloroacetic acid. Acetic acid was determined after steam distillation by titration to pH 7.0, AMC by the methods of Westerfeld (14) and Desnuelle and Naudet (15), diacetyl according to Prill and Hammer (16) and White and coworkers (17), pyruvate by decarboxylation with yeast carboxylase, and acetaldehyde according to Stotz (18). α-Acetolactate does not produce a color typical for acyloins in the Westerfeld procedure (14). However, acidification of a solution of α-acetolactate results in its rapid decarboxylation to AMC (12). Thus application of the Westerfeld test to samples before and after treatment with dilute acid permits the determination of α-acetolactate.

Sodium pyruvate was prepared by neutralizing distilled pyruvic acid with sodium bicarbonate in the cold, DPT was obtained from the Nutritional Biochemicals Corporation, and yeast carboxylase was prepared by an unpublished method.

In the chemical synthesis of DAMC, methylacetylacetone (19) was oxidized with lead tetraacetate and the acetoxymethylacetylacetone was hydrolyzed to DAMC and acetic acid. Thus the method was similar to that described by Krampitz (20) for synthesis of α-acetolactate from methyl-substituted ethyl acetoacetate. A yield of 25.2 gm. of acetoxymethyl-

acetylacetone was obtained from 74.5 gm. of methylacetylacetone. Final purification was accomplished by fractional distillation at 72–80° at a pressure of 5 to 6 mm. Water solutions of DAMC were obtained by hydrolysis of the acetoxy compound in 0.3 N sulfuric acid at room temperature for 1 week. Alkaline hydrolysis was avoided since DAMC is unstable under these conditions. In Table I is given the activity of DAMC in various chemical tests for acyloins, and the reactivity of DAMC with that of AMC, diacetyl, and 2,3-butanediol, compounds generally present in systems forming DAMC, is compared.

TABLE I

Methods for Determining Acyloin Condensation Products and Related Compounds

The figures in the table represent equivalents in the given method for the various compounds when compared with the reaction obtained with an equivalent of the compound for which the test was originally devised.

Method	AMC	DAMC	Diacetyl	2,3-Butanediol
Westerfeld (14)	1.0	1.0	1.0	0
White *et al.* (17)	0	1.0*	1.0	0
Prill and Hammer (16)	0	0	1.0	0
CH_3COOH after HIO_4 oxidation†.	1.0	3.0	2.0	0
CH_3CHO after HIO_4 oxidation†..	1.0	0	0	2.0

* No reaction occurs without NH_2OH for DAMC; NH_2OH is not required for the determination of diacetyl, however.

† Volatile acid determined after periodate oxidation (21). Acetaldehyde determined after periodate oxidation according to Desnuelle and Naudet (15) without previous distillation.

In order to determine the K_m value for AMC formation from pyruvate alone with the use of the pigeon enzyme, it was necessary to use reaction volumes as large as 50 ml. to permit production of sufficient AMC for assay purposes. The reaction was stopped by acidification with dilute sulfuric acid and the AMC concentrated to 10 ml. by saturating each sample with $(NH_4)_2SO_4$ and distilling over one-fifth the original volume. This salting out procedure resulted in quantitative recovery of AMC, but destroyed any diacetyl or DAMC present. Both DAMC and AMC may be concentrated by saturating with NaCl and distilling over one-third the original volume.

Results

Anaerobic Pyruvate Degradations by Pyruvic Oxidase Preparations—
Under anaerobic conditions pyruvate is quantitatively converted to AMC

and α-acetolactate by pigeon breast muscle extracts, as seen in Fig. 1. Both of these condensation products are formed linearly with time. Most bacteria produce AMC through the intermediate formation and subsequent decarboxylation of α-acetolactate (12, 22):
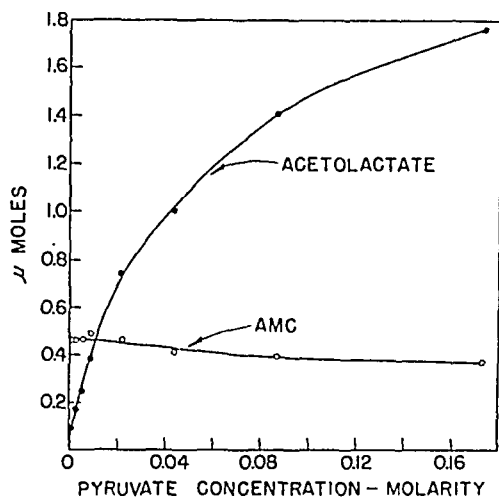
(2) $\qquad 2CH_3COCOOH \rightarrow CH_3C(OH)(COCH_3)COOH + CO_2$

(3) $\qquad CH_3C(OH)(COCH_3)COOH \rightarrow CH_3CHOHCOCH_3 + CO_2$
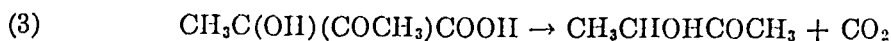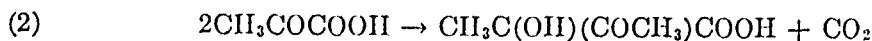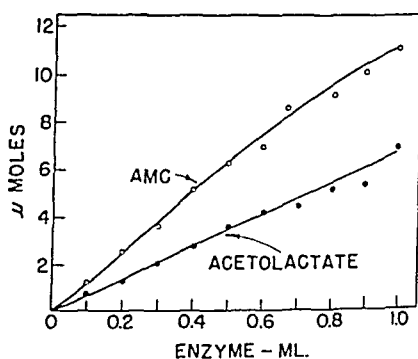

FIG. 1   FIG. 2

FIG. 1. Rate of formation of AMC and α-acetolactate as a function of pyruvate concentration by pigeon breast muscle pyruvic oxidase. Each vessel contained 90 μmoles of potassium phosphate buffer, pH 6.4, 50 γ of DPT, 0.1 ml. of 0.5 per cent MgSO$_4$, varying amounts of sodium pyruvate, and 0.2 ml. of pigeon breast muscle enzyme in a total volume of 1.6 ml. Incubation in air; 60 minutes at 30°.

FIG. 2. Rate of formation of AMC and α-acetolactate as a function of enzyme concentration by pigeon breast muscle pyruvic oxidase. Each vessel contained 100 μmoles of potassium phosphate buffer, pH 7.5, 50 γ of DPT, 10 μmoles of sodium pyruvate, and varying amounts of pigeon breast muscle enzyme in a total volume of 1.6 ml. Incubation in 100 per cent nitrogen; 250 minutes at 30°.

DPT and a divalent cation such as magnesium are required for acyloin condensation, so that an aldehyde-DPT-magnesium complex could account for these reactions. Pyruvate could serve as the acceptor of this "activated" acetaldehyde with the formation of α-acetolactate.

α-Acetolactate cannot be an intermediate in AMC formation by mammalian tissue extracts since these do not contain an α-acetolactic decarboxylase (Equation 3) (21). It has been proposed (8, 23) that AMC is formed by a condensation of two "activated" acetaldehyde units, each attached to a different enzyme molecule. In the presence of excess pyruvate, DPT, and magnesium, all enzyme molecules would appear as "activated" units and the rate of AMC formation would be proportional to the

square of the enzyme concentration. However, our results (Fig. 2) show that the rate of AMC formation is essentially a linear function of enzyme concentration over a 10-fold range.

These data can be explained by assuming that the "activated" acetaldehyde produced during pyruvate decarboxylation is, in part, converted to free acetaldehyde, which could then condense with more "activated" acetaldehyde to give AMC. It is well known that addition of free acetaldehyde accelerates the rate of pyruvate decarboxylation with a corresponding increase in the rate of AMC production (4). The rate of formation of AMC must therefore be equal to the rate of formation of free acetaldehyde, since free acetaldehyde does not accumulate as an end-product of the reaction.

TABLE II

Trapping Free Acetaldehyde Formed from Pyruvate by Pigeon Breast Muscle Enzyme

The complete system contained 1 mmole of sodium pyruvate, 0.5 mmole of potassium phosphate buffer, pH 7.5, 0.25 mg. of DPT, and 0.1 ml. of pigeon breast muscle enzyme; final volume, 8.0 ml. Incubation for 5 hours at 30°. The atmosphere was 100 per cent nitrogen. In Experiment A, the solution was gassed continuously and the acetaldehyde trapped in an ice-cold 2 per cent sodium bisulfite solution; in Experiment B, the vessel was not gassed continuously. The values are given in micromoles.

Experiment	α-Acetolactate	AMC	Acetaldehyde
A	0.63	0.31	0.27
B	0.67	0.60	

Direct evidence for the formation of free acetaldehyde is provided by the data in Table II. Experiment A differs from Experiment B in that the reaction mixture in Experiment A was continuously gassed by a stream of nitrogen which was then passed through a trap containing 2 per cent sodium bisulfite solution. The amount of free acetaldehyde trapped corresponds to the decrease in AMC observed when compared with the amount of AMC formed in Experiment B, which was not continuously gassed. The amount of α-acetolactate formed was the same in both experiments.

Fig. 3 shows the effect of acetaldehyde concentration on AMC formation in the presence of a concentration of pyruvic acid small enough so that virtually no α-acetolactate is formed. There is a small but definite amount of AMC produced in the absence of added acetaldehyde. With a sufficiently high concentration of acetaldehyde there is as much as a 12-fold stimulation of AMC production. The K_m for AMC formation for varying concentrations of acetaldehyde and a fixed pyruvate concentration is

3.4×10^{-4} M. Extrapolation of a Lineweaver-Burk plot of these data gives a value of 2.2×10^{-5} M for the concentration of free acetaldehyde
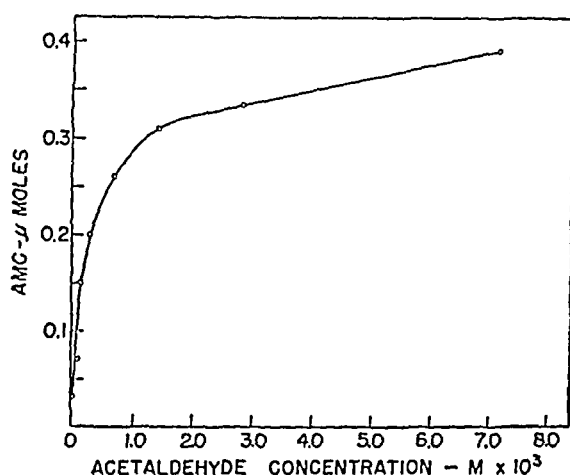

FIG. 3. Rate of formation of AMC as a function of acetaldehyde concentration by pigeon breast muscle pyruvic oxidase. Each vessel contained 6.0 ml. of 0.1 M potassium phosphate buffer, pH 6.4, 0.25 mg. of DPT, 0.5 ml. of 0.5 per cent $MgSO_4$, 8 μmoles of sodium pyruvate, 0.1 ml. of pigeon breast muscle enzyme, and varying amounts of acetaldehyde; total volume, 8.0 ml. Incubation in air; 30 minutes at 30°.

TABLE III

Formation of Racemic α-Acetolactic Acid from Pyruvic Acid by Pyruvic Acid Oxidase Preparations

The basal system contained 100 μmoles of potassium phosphate buffer, pH 5.9, 50 γ of DPT, 16 μmoles of sodium pyruvate, and 0.5 ml. of pigeon breast muscle or *E. coli* enzyme; total volume, 1.6 ml. 0.3 ml. of α-acetolactic decarboxylase, either initially or after the indicated times, was added. Incubation in 100 per cent nitrogen at 30°.

Experiment No.	Preparation	Total incubation time	Time with acetolactic decarboxylase	AMC	Acetolactate	AMC after acetolactic decarboxylase
		hrs.	*hrs.*	*μmoles*	*μmoles*	*μmoles*
1	Pigeon	3.5	0	5.23	4.57	
	"	3.5	3.5	7.17	2.2	1.94
2	"	3.5	0	4.9	5.1	
	"	3.5	0.75	7.4	2.3	2.5
3	*E. coli*	1.75	0	0.11	0.58	
	" "	1.75	0.25	0.40	0.26	0.29

formed from pyruvate as the only substrate. The K_m value for AMC production from pyruvate alone is 1.1×10^{-5} M. This compares with a value of 2×10^{-5} M obtained by Jagannathan and Schweet (11), using the same enzyme to measure the oxidation of pyruvate.

The optimal concentration of pyruvate for AMC formation, in the absence of added acetaldehyde, occurs at a level at which pyruvate itself is a very poor acceptor of "activated" acetaldehyde (Fig. 1). The fact that "activated" acetaldehyde can condense with pyruvate to form α-acetolactate may be regarded as evidence that there is a single condensing enzyme involved, which is not absolutely specific with respect to acceptors. This idea is supported by the fact that the α-acetolactate formed in these systems is a racemic mixture. As can be seen in Table III, addition of bacterial α-acetolactic decarboxylase (12) results in decarboxylation of only one-half of the α-acetolactate formed when the decarboxylase is added either initially (Experiment 1) or for only a short time before the conclu-

TABLE IV

Formation of Condensation Products at Various Stages of Purification of Pigeon Pyruvic Oxidase

Each vessel contained 100 μmoles of potassium phosphate buffer, pH 7.5, 50 γ of DPT, 35 μmoles of sodium pyruvate, and 0.5 ml. of the respective enzyme fraction; total volume, 1.6 ml. Incubation for 3.5 hours in 100 per cent nitrogen at 30°.

Purification step*	AMC	Acetolactate	$\dfrac{AMC}{Acetolactate}$
	μmoles	μmoles	
2	4.6	6.1	0.755
4	1.54	2.46	0.625
6†	1.36	1.86	0.73
6‡	2.34	3.60	0.65

* The purification steps are those of Jagannathan and Schweet (11).
† First precipitate fraction.
‡ Purest fraction.

sion of the experiment (Experiment 2). It has been shown (12) that the bacterial α-acetolactic decarboxylase is specific for the dextrorotatory isomer of α-acetolactate. That the α-acetolactate formed with *E. coli* extract is also the racemic mixture is seen in Table III (Experiment 3).

The K_m value for pyruvic acid obtained from a Lineweaver-Burk plot of the data of Fig. 1 for α-acetolactate formation is 0.047 M and compares with a K_m of 0.037 M for the bacterial α-acetolactate-forming system (12). The rate of AMC formation remains essentially constant with increasing pyruvate concentrations above the optimal value. The slight drop in AMC production may be accounted for by increased competition of pyruvic acid with free acetaldehyde for the "activated" aldehyde.

According to the results in Table IV, both AMC and α-acetolactate formation are mediated by the homogeneous pyruvic oxidase from pigeon breast muscle, as well as by crude fractions from the same source. Thus there is no evidence that separate condensing enzymes are involved.

Anaerobic Diacetyl Degradations by Pyruvic Oxidase Preparations—Diacetyl has been shown to serve as a substrate with several pyruvic acid oxidases (8, 24). When it is the substrate, a mixture of AMC and DAMC results. The production of these condensation products as a function of diacetyl concentrations is presented in Fig. 4. DAMC bears the same relationship to α-acetolactate as diacetyl does to pyruvate, and the reactions with diacetyl are in every way completely analogous to those with pyruvate as substrate. AMC is apparently formed in the same manner as
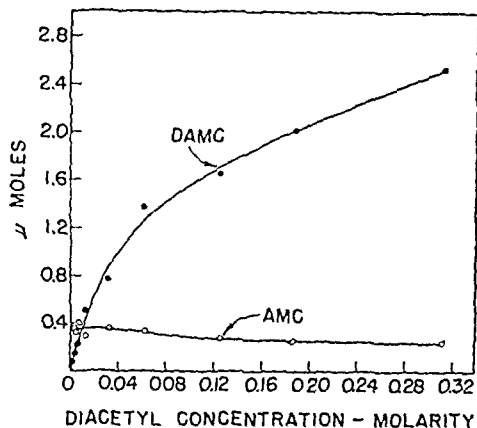

DIACETYL CONCENTRATION – MOLARITY
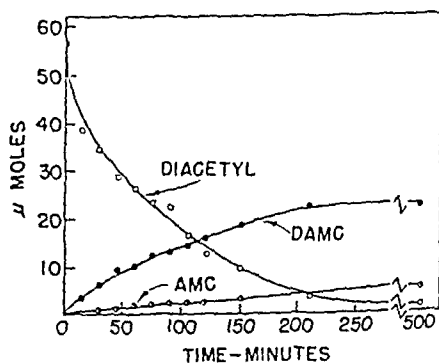FIG. 4

TIME–MINUTES
FIG. 5

FIG. 4. Rate of formation of AMC and DAMC as a function of diacetyl concentration by pigeon breast muscle pyruvic oxidase. Each vessel contained 50 μmoles of sodium bicarbonate, 50 γ of DPT, 0.1 ml. of 0.5 per cent MgSO$_4$, 0.2 ml. of pigeon breast muscle enzyme, and varying amounts of diacetyl; total volume, 1.6 ml. Incubation for 60 minutes in 100 per cent CO$_2$ at 30°. The diacetyl was added after gassing to avoid loss due to volatilization.

FIG. 5. Anaerobic dissimilation of diacetyl as a function of time by pigeon breast muscle pyruvic oxidase. Each vessel contained 60 μmoles of sodium bicarbonate, 50 γ of DPT, 0.1 ml. of 0.5 per cent MgSO$_4$, 57 μmoles of diacetyl, and 0.5 ml. of pigeon breast muscle enzyme; total volume, 1.6 ml. Incubation in 100 per cent CO$_2$ at 30°.

with pyruvate. DAMC is not an intermediate in AMC formation and is not hydrolyzed to AMC in this system. Fig. 5 presents a time curve for the utilization of diacetyl and the concomitant formation of condensation products. Further data on how the ratio of AMC to DAMC depends upon the diacetyl concentration are given in Table V. For both the amounts used, diacetyl was added directly to the reaction mixture in one vessel and in the other simply allowed to distil from a side arm. In each case the experiment was continued until all the diacetyl had been used. When diacetyl is permitted to distil into the reaction flask so that the concentration of diacetyl in the reaction mixture never becomes very great, the chief end-products are then AMC and acetic acid. Here the balance

obtained approaches that for the diacetyl mutase reaction (9) (Equation 4).

(4) $2CH_3COCOCH_3 + 2H_2O \rightarrow CH_3CHOHCOCH_3 + 2CH_3COOH$
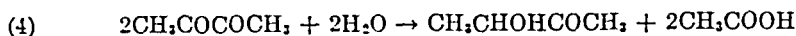
Under alkaline conditions diacetyl rapidly dimerizes (25). Thus after 110 minutes at 38° and pH 7.5, only 38.7 per cent of the starting diacetyl

TABLE V

Effect of Diacetyl Concentration on AMC-DAMC Ratio

Each vessel contained 50 μmoles of sodium bicarbonate, 25 γ of DPT, 0.1 ml. of 0.5 per cent $MgSO_4$, 0.7 ml. of pigeon breast muscle enzyme, and the indicated amounts of diacetyl; final volume, 1.6 ml. Incubation in 100 per cent CO_2 for 10 hours at 30°.

Diacetyl	Method of addition*	AMC	DAMC	$2 \times$ (AMC + DAMC)	$\dfrac{AMC}{DAMC}$
μmoles		*μmoles*	*μmoles*	*μmoles*	
16.3	Direct	3.6	4.3	15.8	0.84
16.3	Distilled	5.9	2.3	16.4	2.56
50.0	Direct	7.2	16.8	48.0	0.43
50.0	Distilled	14.6	10.7	50.6	1.36

* See the text for details.

TABLE VI

Balance Studies for Degradation of Diacetyl by Pigeon Breast Muscle Enzyme

Each reaction vessel contained 400 μmoles of sodium bicarbonate, 0.25 mg. of DPT, 0.5 ml. of 0.5 per cent $MgSO_4$, 3.0 ml. of pigeon breast muscle enzyme, and the indicated amounts of diacetyl; total volume, 8.4 ml. Incubation in 100 per cent CO_2 for 12 hours at 30°. The values are given in micromoles.

Initial diacetyl	Diacetyl used	$2 \times$ (AMC + DAMC)	AMC	DAMC	Acetic acid	$2 \times$ (AMC + DAMC)
195	192	205.6	24.4	78.4	128.6	127.2
374	368	362.6	37.3	144.0	228.4	218.6

remains unpolymerized. It seems probable that other investigators, unaware of this phenomenon, may have experienced artifacts when using diacetyl as a substrate. This point will be discussed more fully below.

Table VI gives the complete balances for AMC, DAMC, and acetic acid formation from diacetyl by pigeon breast muscle enzyme. As indicated above, the production of 1 mole of either AMC or DAMC requires 2 moles of diacetyl.

Experiments with Other Pyruvic Acid Enzymes—It has been seen (5, 12) that different pyruvate enzymes can utilize pyruvate as either, or both,

donor or acceptor of "activated" aldehyde. The pyruvic carboxylase from pig heart (4) forms AMC and α-acetolactate from pyruvate and AMC, and DAMC and acetic acid from diacetyl (Table VII, Experiments 1 and 2). A crude sonic extract of *E. coli* able to dismute pyruvate to acetate, lactate, and CO_2 also forms α-acetolactate (Table III). In the presence of 0.06 M sodium pyruvate, 0.7 μmole of α-acetolactate and 7.7 μmole of CO_2 were formed in 1 hour at 30° with 0.5 ml. of this preparation.

TABLE VII

Action of Pig Heart and Pigeon Breast Muscle Enzymes on Pyruvate and Diacetyl

Experiment 1: The reaction mixture contained 100 μmoles of potassium phosphate buffer, pH 7.5, 25 γ of DPT, 0.1 ml. of 0.5 per cent $MgSO_4$, 28.2 μmoles of sodium pyruvate, and 0.5 ml. of pig heart enzyme; total volume, 1.6 ml.; incubation for 8 hours in 100 per cent nitrogen at 30°. Experiment 2: Each vessel contained 70 μmoles of sodium bicarbonate, 50 γ of DPT, 0.1 ml. of 0.5 per cent $MgSO_4$, 100 μmoles of sodium pyruvate or 26.7 μmoles of diacetyl, and 0.5 ml. of pig heart enzyme; total volume, 1.6 ml.; incubation for 4 hours in 100 per cent CO_2 at 30°. Experiment 3: Each vessel contained 60 μmoles of sodium bicarbonate, 50 γ of DPT, 0.1 ml. of 0.5 per cent $MgSO_4$, 50 μmoles of sodium pyruvate or 23.7 μmoles of diacetyl, and 0.5 ml. of pigeon breast muscle enzyme; total volume, 1.6 ml.; incubation for 2.5 hours in 100 per cent CO_2 at 30°. The values are given in micromoles.

Experiment No.	Substrate	AMC	Acetolac-tate	AMC + ace-tolactate	DAMC
1	Pyruvate	3.1	4.4	7.5	
2	"			2.2	
	Diacetyl	1.6			12.1
	Pyruvate + diacetyl			1.8	16.8
3	Pyruvate			4.9	
	Diacetyl	2.5			8.3
	Pyruvate + diacetyl			4.8	11.4

With 0.06 M diacetyl as substrate, 1.9 μmoles of DAMC were formed in 1 hour at 30°.

Cells of *Streptococcus faecalis* (strain 10C1) have been found to contain a potent α-acetolactate-forming system in addition to an α-acetolactic decarboxylase typical of most bacteria that form AMC (22). When diacetyl was tested with cell suspensions of this organism, DAMC was produced. That DAMC formation was here due to the presence of pyruvic oxidase, and not to the specific bacterial α-acetolactate-forming enzymes, was indicated by the further observation that no DAMC could be synthesized from diacetyl with an α-acetolactate-forming cell-free extract from *A. aerogenes*, which contains no pyruvic oxidase activity (12). After yeast carboxylase dissimilation of pyruvate, no α-acetolactate could be detected, even when

trapping agents such as sodium bisulfite and dimedon were added to remove free acetaldehyde. Diacetyl alone was inactive as a substrate for the yeast enzyme but could function as acceptor of "activated" aldehyde, with the formation of DAMC, when pyruvate was added as the "activated" aldehyde donor.

Apparent Competitive Effects of "Activated" Acetaldehyde Acceptors—The "activated" aldehyde formed by pyruvic oxidase is normally further oxidized, presumably via steps involving lipoic acid (26). Therefore, under conditions by which oxidation can occur, there should be a decrease in the rate of formation of condensation products such as has been demonstrated by Schweet and coworkers (8, 11). Our studies have confirmed these results and also show that acetaldehyde can compete with an oxidizing system (ferricyanide) for "activated" aldehyde, thereby completely suppressing pyruvate oxidation without impairing AMC production. This may account for the reported inability of the pigeon breast muscle enzyme to oxidize acetaldehyde (11). Presumably here, too, acetaldehyde competes for "activated" acetaldehyde to a sufficient extent to suppress its own oxidation completely.

The importance of acetaldehyde as an acceptor could also be demonstrated by the effect of increasing concentrations of acetaldehyde on α-acetolactate formation from pyruvate with pigeon breast muscle enzyme. With a pyruvate concentration high enough to give 2.5 times as much α-acetolactate as AMC from pyruvate alone (Fig. 1), a concentration of acetaldehyde of 7×10^{-4} M was sufficient to inhibit the rate of α-acetolactate formation by 50 per cent. At 3×10^{-3} M acetaldehyde no α-acetolactate could be detected. The effect of increasing acetaldehyde concentration on AMC formation is given in Fig. 3.

When pyruvate or diacetyl is the sole substrate, the concentration of free acetaldehyde is always constant and very small, as shown above. In these cases increased pyruvate or diacetyl concentrations decreased AMC formation only slightly (Figs. 1 and 4), since these compounds are relatively poor acceptors of "activated" aldehyde. It should be noted that the specific rotation of AMC produced by animal carboxylases from pyruvate and acetaldehyde (α_D $-84°$) is as high as that of AMC produced by bacteria via the decarboxylation of optically active α-acetolactate, although α-acetolactate produced by pyruvic oxidase lacks optical specificity.

Schweet and coworkers (8) have made a report, which we have confirmed, that pyruvic oxidase from pigeon breast muscle will preferentially dissimilate pyruvate in the presence of both pyruvate and diacetyl. The results of our experiments with mixed substrates, however, indicate that DAMC rather than AMC is the chief product formed, diacetyl acting as acceptor of "activated" aldehyde (Table VII, Experiments 2 and 3). The

amounts of AMC and α-acetolactate formed are nearly the same or even less than with pyruvate alone.

pH Optima—The optimal pH for the formation of condensation products from pyruvate and diacetyl is approximately 6.5. The data for pyruvate as the substrate are given in Fig. 6. Virtually identical activity and optimal pH values were obtained with either bicarbonate or phosphate buffer.

Derivatives of DAMC—Solutions of DAMC form a derivative with 2,4-dinitrophenylhydrazine in the cold. *Derivatives of enzymatically and chemically synthesized samples of DAMC were purified by recrystallization from dioxane. The melting point of all of the products is 234-238°*
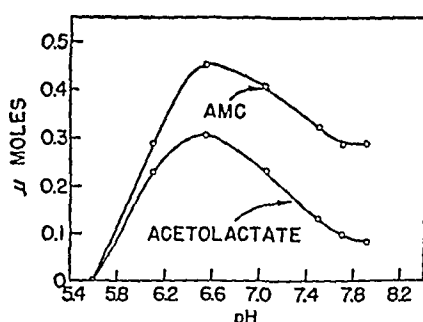
FIG. 6. Optimal pH for the formation of condensation products by pigeon breast muscle enzyme. Each vessel contained 100 μmoles of potassium phosphate buffer at the indicated pH, 50 γ of DPT, 0.1 ml. of 0.5 per cent $MgSO_4$, 20 μmoles of sodium pyruvate, and 0.2 ml. of pigeon breast muscle enzyme. Total volume, 1.6 ml. Incubation in air for 1 hour at 30°. The pH of each sample was measured in a pH meter at the conclusion of the experiment.

(uncorrected). Mixtures of the derivatives did not have depressed melting points.

$C_{18}N_8O_9H_{18}$.

Calculated.	C 44.09, H 3.67, N 22.85
Found, derivative of chemically synthesized DAMC	C 44.19, H 3.83, N 22.61
Found, derivative of DAMC produced by pigeon enzyme	C 43.97, H 3.41, N 22.55

DISCUSSION

Although this is the first report dealing with the formation of α-acetolactate from pyruvate by animal enzymes, evidence for this reaction in pig heart can be found in the work of Green and coworkers (4). They noted that an approximately 1:1 ratio of CO_2 liberated to pyruvate utilized occurred only when the initial concentration of pyruvate was not very high. At higher pyruvate concentrations, less than the expected amount of CO_2 was liberated. This can now be explained as due to increased formation

of α-acetolactate at high pyruvate concentrations with a consequent decrease in CO_2 liberated (Equation 2). Addition of acetaldehyde to these latter preparations results in a 4- to 5-fold increase in the rate of CO_2 evolution (4) since the added acetaldehyde competes very effectively with the pyruvate as an "activated" aldehyde acceptor. It should be noted that it is only the net increase in acceptor resulting from acetaldehyde addition that is manifested in this more rapid CO_2 evolution (see Fig. 3).

Schweet and coworkers (8) have concluded that the diacetyl mutase enzyme is identical with pyruvic oxidase in pigeon breast muscle. The occurrence of such a reaction (Equation 4) has not been substantiated by the results presented above. AMC is actually a relatively minor endproduct in this reaction when sufficient concentrations of diacetyl are employed. The results of Green and coworkers (9) and Schweet and coworkers (8) may have been due to several obscuring factors. DAMC reacts as diacetyl in the tests employed by these workers (Table I). As shown above, diacetyl dimerizes rapidly at pH 7.5 and 38°, the conditions employed in the original diacetyl mutase experiments. Assays for acetic acid, by the method of Black (27), were made by Schweet and coworkers (8). Since DAMC distils with water and DAMC is hydrolyzed to AMC and acetic acid under alkaline conditions, assays for acetic acid and AMC in such samples should be correspondingly high.

Thus there is no evidence for a dismutation in the diacetyl mutase reaction. The rate of formation of AMC from diacetyl is no faster than the corresponding rate from pyruvate. In view of the formation of free acetaldehyde discussed above, most, if not all, of the AMC synthesized can be accounted for on the basis of condensation of free acetaldehyde with "activated" aldehyde. The fact that anaerobic experiments with some pyruvic oxidase preparations display differences in the ratios of AMC-α-acetolactate produced can be interpreted as attributable to differences in the ability of these systems to dissociate free acetaldehyde. Liberation of such free acetaldehyde may be a consequence of partial dissociation of the acetaldehyde-DPT complex from the enzyme with subsequent non-enzymatic release of free acetaldehyde and DPT. Further evidence supporting this view will be presented in a future publication. Other DPT enzymes such as the α-ketoglutaric oxidase also appear partially to dissociate an "activated" complex with subsequent breakdown to succinic semialdehyde (4, 28). Free acetaldehyde has also been detected with various animal and bacterial preparations by many workers using trapping agents (29–33).

SUMMARY

1. Under anaerobic conditions, pyruvic oxidase preparations from animal tissues and bacteria can form α-acetolactate as well as AMC from pyruvate.

2. AMC formation is a consequence of the production of free acetaldehyde, which then can serve as acceptor for "activated" acetaldehyde derived from pyruvate.

3. α-Acetolactate formed by pyruvic oxidase is a racemic mixture.

4. Addition of acetaldehyde can stimulate AMC formation from pyruvate as much as 12-fold and inhibits the synthesis of α-acetolactate.

5. DAMC and AMC are formed simultaneously when diacetyl is the substrate for pyruvic oxidase.

6. A method is described for the chemical synthesis of DAMC.

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SYNTHESIS IN VITRO AND IN VIVO OF CO[60] CONTAINING VITAMIN B₁₂-ACTIVE SUBSTANCES BY RUMEN MICROORGANISMS*

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The importance of dietary cobalt for the growth and well being of ruminants has been well established (1, 2). Since the discovery of cobalt in the vitamin B_{12} molecule, several workers (3–8) have reported results indicating that the primary function of cobalt in the ration of ruminants is to enable the rumen microorganisms to synthesize vitamin B_{12}.

Changes in the chemical structure of vitamin B_{12} can occur (9–11) by replacement of the cyanide group of true vitamin B_{12} (cyanocobalamin) with other radicals: vitamin B_{12b} (hydroxy group), vitamin B_{12c} (nitrite group). Other compounds have been discovered which possess varying degrees of vitamin B_{12} activity but exhibit greater differences in structure. Among these are vitamins B_{12m} (12), B_{12f} (13), pseudovitamins B_{12} and B_{12d} (14), and factors A, B, and C (15, 16). All of these substances were produced by microorganisms inhabiting the gastrointestinal tracts of animals. Many of these compounds were found to be relatively inactive in higher animals.

Bentley *et al.* (7), in this laboratory, found that the addition of cobalt to poor roughage rations for steers improved the rate of growth and increased the vitamin B_{12} activity of the rumen liquor and the livers 5- to 10-fold. Since ruminant animals depend on microbial synthesis for their vitamin B_{12}, it became of interest to investigate the nature of the vitamin B_{12} produced. The results reported herein describe the separation of vitamin B_{12}-active substances synthesized by rumen microorganisms growing *in vitro* and *in vivo* in the presence of Co^{60}.

EXPERIMENTAL

Rumen Fermentation in Vitro—Rumen microorganisms were cultured *in vitro* on a semipurified medium according to the procedure described

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by Bentley *et al.* (17). The basal medium consisted of cellulose,[1] urea, glucose, minerals, valeric acid, biotin, and *p*-aminobenzoic acid. Strained rumen liquor from steers (obtained through a rumen fistula) was centrifuged in a Sharples supercentrifuge and the sediment of microflora resuspended in phosphate buffer, pH 7.0. This suspension was used as the inoculum for the fermentations.

Rumen Studies in Vivo—A 90 pound wether lamb was placed in a metabolism crate constructed to allow separate collection of the feces and urine. Co^{60} was injected into the rumen cavity of the animal directly through the left flank with a 10 ml. hypodermic syringe equipped with a 6 inch No. 16 gage needle. Injections of 22 γ of Co containing 90 μc. of Co^{60} each were made daily over a period of 4 days. The feces were collected in a bag attached to the animal by a web harness and lined with plastic. On the 5th day after the first injection, the animal was sacrificed, and the liver, kidneys, and rumen contents were removed for analysis.

Paper Electrophoretic Separations—The fermentation flask contents as well as the rumen contents and feces from the intact animal were dried at 50° in a forced draft oven and ground to a coarse powder with a mortar and pestle. 3 to 5 gm. portions of these samples were extracted in hot water and centrifuged. Since this crude water extract had a high concentration of dissolved solids and a low concentration of vitamin B$_{12}$, it did not lend itself to proper paper electrophoretic separation. To clarify the extract further, the supernatant fluid was stirred with 20 gm. of activated charcoal (Nuchar C-190-N or Darco G-60), and the suspension was filtered on a Büchner funnel. The vitamin B$_{12}$-active substances were eluted with hot 70 per cent acetone and the eluates concentrated under reduced pressure to 5 to 15 ml. Fresh samples of the sheep liver and kidney were extracted in hot water, and the extract was similarly treated with charcoal, eluted, and concentrated.

A paper electrophoretic technique similar to that reported by Holdsworth *et al.* (18) was used to separate the vitamin B$_{12}$-active substances. Aliquots[2] of the above samples were dried as spots on 4 × 40 cm. strips of filter paper (Schleicher and Schuell No. 2043-B or Whatman No. 1). After wetting the strips with 2 N acetic acid, they were suspended in a horizontal cassette,[3] the ends dipping into the electrode baths containing 2 N acetic acid. A potential of 8.5 volts per cm. at a current flow of 4 to 5 ma. was applied for 12 hours.

Vitamin B$_{12}$ Assays—Both *Lactobacillus leichmannii* ATCC 7830 and

[1] Solka-Floc No. 40A, Brown Company, Berlin, New Hampshire.

[2] With some samples, several aliquots were necessary to obtain sufficient concentrations for detection. In this case, a heat lamp was used to hasten the drying process.

[3] LKB paper electrophoresis equipment, Ivan Sorvall, Inc., Norwalk, Connecticut.

Escherichia coli ATCC 11105 were used to assay vitamin B_{12} activity. For the *L. leichmannii* assays, the titrimetric method was used with a 72 hour incubation. The procedure was a combination of those reported by Skeggs *et al.* (19) and Thompson *et al.* (20) and was described in detail by Moinuddin and Bentley (21).

For the *E. coli* assays, the medium used was that described by Davis and Mingioli (22) with the addition of 0.05 per cent NaCl, as suggested by Johansson (23). The organism was maintained on agar slants prepared as described by Harrison *et al.* (24).

Detection of Separation—Agar plates consisted of double strength *E. coli* medium plus 0.3 per cent sodium propionate (to prevent mold growth) and 2 per cent agar and were inoculated with a saline-washed culture. Paper strips were dried at room temperature and placed on the plates. After incubation for 12 hours at 37°, zones of growth indicated the position of the vitamin B_{12}-active substances on the paper.

Detection of Radioactivity—Co^{60} activity was measured by drying samples in nickel planchets and counting with a Geiger-Müller tube (2.6 mg. per sq. cm. mica window) attached to a Berkely decimal scaler, model 2000.

Radioautographs were made by exposing type K x-ray film to the electrophoretic strips.

Isotope—Co^{60} was obtained from the Oak Ridge National Laboratory.

Results

Studies in Vitro—In the first study, seven identical rumen fermentations were prepared. To each, except the zero time flask, 5.5 γ of cobalt (as $CoCl_2$) containing 22.5 μc. of Co^{60} activity were added. At intervals of 6, 12, 24, 48, 72, and 96 hours one flask was removed, and the contents were prepared as described above.

Fig. 1 illustrates the radioautographs of paper electrophoretic separation performed on both the crude water extracts and charcoal eluates of two samples of rumen contents *in vitro*. Three distinct zones of Co^{60} activity appeared in the separation performed on crude water extracts and were labeled Substances 1, 2, and 3. The remainder of the activity appeared as a diffuse streak. Charcoal clarification, however, permitted this diffuse portion to separate distinctly into four zones of activity, Substances 4, 5, 6, and 7. The electrophoretic mobilities of these spots on Schleicher and Schuell No. 2043-B paper were 0, 0.57 and 1.25 (Substance 2 was actually a double zone), 2.21, 2.77, 3.67, 4.52, and 5.08 ($\times 10^{-5}$ sq. cm. volt^{-1} sec.$^{-1}$), respectively. Since the pH of the electrolyte was between 2.2 and 2.3, electroosmotic effects were presumed to be negligible (25).

E. coli bioautographs of similar strips as well as later titrimetric analyses

revealed that all the Co⁶⁰-active zones contained vitamin B_{12} activity. Separation of crystalline vitamin B_{12} samples known to contain some vitamin B_{12b} showed that Substance 4 was true vitamin B_{12} (cyanocobalamin) and Substance 6 was vitamin B_{12b} (hydroxycobalamin).

Substances 2 and 3 did not appear in the charcoal eluate. It is possible they were not eluted from the charcoal with 70 per cent acetone. Though protein-bound forms of vitamin B_{12} are known to exist, Substances 2 and 3 were not of this type, since incubation with proteolytic enzymes did not change their mobilities.
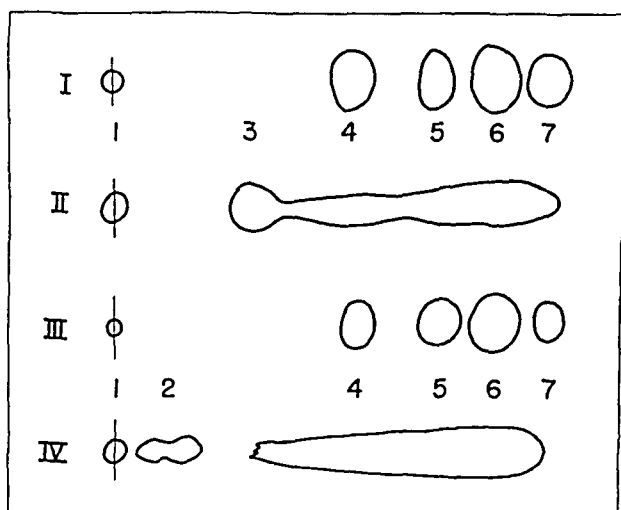
FIG. 1. Diagram of radioautograph of paper electrophoretic separation of Co⁶⁰-active substances in rumen liquor. The Co⁶⁰ zone (No. I) represents the starting point. Zone I, 48 hours, charcoal eluate; Zone II, 48 hours, crude water extract; Zone III, 72 hours, charcoal eluate; Zone IV, 72 hours, crude water extract. The arabic numbers indicate the substances discussed in the text.

The position of the seven vitamin B_{12}-active substances in similar strips was located by radioautography, and the strips were cut into sections containing the seven substances. Each section was extracted with hot water and assayed titrimetrically for vitamin B_{12} activity by the *L. leichmannii* assay. The percentages contributed by each substance to the total activity of the samples are presented in Table I.

Vitamins B_{12} and B_{12b} together[4] contributed 50 to 60 per cent of the total vitamin B_{12} (*L. leichmannii*) activity, while Substance 5 contributed between 30 and 40 per cent. Of Substances 1, 2, 3, and 7, only Substances 3 and 7 occurred in significant amounts. The highest values obtained for Substances 3 and 7 were 9.2 and 12.1 per cent, respectively.

[4] Since vitamin B_{12} and vitamin B_{12b} are interconvertible merely by the removal or addition of the cyanide group, they can be considered together.

Similar strips were sectioned, the individual sections were extracted, and the Co^{60} activity was measured by counting aliquots in planchets. Table II presents a comparison of the distribution of vitamin B_{12} activity and Co^{60} activity among the various substances in a typical sample.

The Co^{60} distribution is slightly different from that of vitamin B_{12}. Substances 3 and 7 have relatively more Co^{60} activity than would be predicted from their vitamin B_{12} activities. This was true in all samples tested and compared in this manner. The data of Table II indicate the

TABLE I

Distribution of L. leichmannii Activity* in Electrophoretic Separation of Vitamin B_{12}-Like Substances in Rumen Liquor in Vitro

	Sub-stance No.	Fermentation time					
		6 hrs.	12 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
Water extracts	1	2.4	2.3	1.6	1.0	1.2	0.6
	2	3.5	4.4	2.6	1.0	$\left\{\begin{array}{l}3.0\dagger\\10.9\end{array}\right.$	1.2
	3	6.0	5.8	9.2	3.8		0.9
Charcoal eluates	4	24.7	16.6	20.2	23.3	23.2	16.5
	5	29.3	31.7	38.6	39.2	33.8	29.9
	6	31.4	38.1	30.3	30.1	34.8	35.0
	7	7.8	8.7	8.6	3.2	2.0	12.1

* Activity is given as per cent of the total vitamin B_{12} activity in the respective electrophoretic paper strips. However, Substances 1, 2, and 3 were determined on separations performed on the crude water extracts, while Substances 4, 5, 6, and 7 were obtained from charcoal-treated samples. Consequently, the activity contributions cannot be added and expected to total 100 per cent.

† In the 72 hour sample, Substance 2 appeared as a double zone and no Substance 3 appeared. In all the other samples, Substance 3 appeared, but no distinct Substance 2 was present. In those cases, the activities given for Substance 2 are the result of tailing of other zones.

Co^{60} contribution from Substance 5 was less than its vitamin B_{12} contribution. However, this difference was not usually as large as indicated. Since the data were calculated as percentages of total activity, the greater activity for Substances 3 and 7 would necessitate the lowering of the relative activity of the other constituents.

In a second experiment, three basal flasks were prepared in the same manner as those in the first study. Additions were made as follows: Flask 1, basal, no additions; Flask 2, basal + 4.5 gm. of starch as a readily fermentable source of carbohydrate; and Flask 3, basal + 4.2 γ of Co (as $CoCl_2$) containing 17 μc. of Co^{60} activity. The flasks were incubated

for 48 hours, and the contents were prepared and sampled as described earlier.

TABLE II

Comparison of Distribution of Vitamin B_{12} and Co^{60} Activity* in Rumen Liquor

Substance No.	Vitamin B₁₂ activity	Co⁶⁰ activity
1†	1.76	2.55
2†	3.55	6.2
3†	9.9	19.2
4‡	26.3	25.7
5‡	38.7	24.0
6‡	27.6	24.8
7‡	2.1	13.3

* The values are given as per cent of the total activity found in an electrophoretic paper strip.

† Data taken from crude water extracts.

‡ Data taken from charcoal eluates.

FIG. 2. Diagram of bioautographs (*E. coli*) of paper electrophoretic separation of vitamin B_{12}-active substances in rumen liquor (second trial). Zone I, basal + starch, charcoal eluate; Zone II, basal + Co^{60}, charcoal eluate; Zone III, basal + starch, crude water extract; Zone IV, basal + Co^{60}, crude water extract.

An *E. coli* bioautograph of separations performed on these samples is illustrated in Fig. 2. Although seven zones of *E. coli* growth did appear, the first two were very faint. The remaining five zones were distinct, although not entirely separated from one another. In these samples a substance not demonstrated in the previous experiment appeared between

Substances 4 and 6, thus placing two substances in this range. For consistency, the two were labeled Substances 5a and 5b; for the remainder the labels by which they were denoted previously were retained; *i.e.*,

<div align="center">

TABLE III

Distribution of Vitamin B_{12} Activity in Vitamin B_{12}-Active Substances Synthesized by Rumen Microorganisms in Second Trial

</div>

Substance No.	Distribution of activity*		
	Flask 1 (basal)	Flask 2 (starch)	Flask 3 (Co⁶⁰)
1	0.9	0.8	0.7
2	1.3	1.1	0.6
4	12.8	13.9	15.1
5a	27.7	26.1	27.6
5b	19.7	17.6	16.9
6	26.3	27.4	31.1
7	8.8	11.0	7.0

* Activity is given as per cent of the total vitamin B_{12} activity in the respective strip. Portions of activity found in other sections of the strip, but not due to a distinct zone, were omitted from the table.
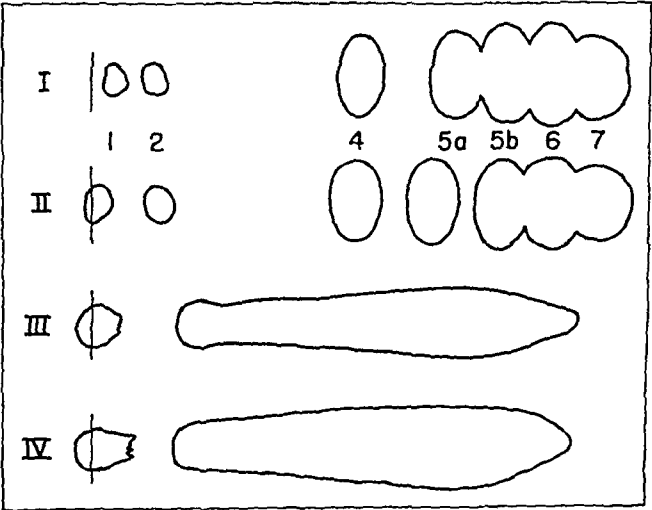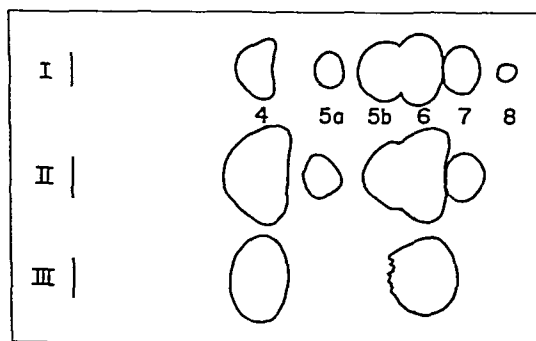

FIG. 3. Diagram of bioautographs (*E. coli*) of paper electrophoretic separation of vitamin B_{12}-active substances in sheep rumen liquor. Zone I, 20 aliquots applied to spot; Zone II, 20 aliquots + crystalline vitamins B_{12} and B_{12b}; Zone III, crystalline vitamins B_{12} and B_{12b}.

Substances 4, 6, and 7. A good separation of zones could not be obtained on the crude water extracts of these samples.

Table III presents the distribution of the vitamin B_{12} activity (*L. leichmannii*) among the various vitamin B_{12}-active substances. Vitamins B_{12} and B_{12b} (Substances 4 and 6) contributed between 39 and 45 per cent of the total activity, which is somewhat less than was found in the first

trial. Substances 5a and 5b both contributed major portions to the total activity. Substance 7, although distinct in the autographs, contributed little to the total *L. leichmannii* activity.

Study in Vivo—Paper electrophoretic separation of the vitamin B_{12}-active substances produced *in vivo* revealed the presence of several substances similar to those found in studies *in vitro*. Fig. 3 illustrates the bioautograph of such a separation performed on the rumen liquor. This autograph demonstrates the presence of five distinct vitamin B_{12}-active substances which correspond to Substances 4, 5a, 5b, 6, and 7 described previously. In especially sensitive bioautographs, zones of growth could be detected in positions previously designated Substances 1 and 2 and

TABLE IV

Distribution* of Vitamin B_{12} Activity (L. leichmannii) in Electrophoretic Separations of Vitamin B_{12}-Active Substances from Sheep Given Co^{60}

Substance No.	Rumen liquor	Feces	Kidney	Liver
1	2.6		2.0	2.5
2		5.2	1.4	
4	29.0	23.3	27.1	44.2
5a	9.6	8.0	6.9	5.2
5b	26.8	21.2	53.5	15.9
6	20.1	29.3	9.1	30.3
7	10.6	12.9		
8	1.3			1.9

* Activity is given as per cent of the total activity in the electrophoretic strip of a given sample.

also in a position beyond Substance 7 (designated Substance 8). At no time were the latter three substances present in major amounts.

Similarly, the feces samples separated into five and occasionally six active entities. Liver and kidney samples both exhibited Substances 4, 5a, 5b, and 6, with traces of Substances 1 and 2.

Strips similar to those autographed above were cut into sections, and the activity of each vitamin B_{12}-active substance was determined by titrimetric tube assay (*L. leichmannii*). Table IV presents the distribution of the total vitamin B_{12} activity among the various vitamin B_{12}-active substances in each sample.

It was demonstrated in the study *in vitro* and again in this experiment (Fig. 3) that Substances 4 and 6 were vitamins B_{12} and B_{12b}, respectively. These substances together contribute only about half of the microbiological activity of both the sheep rumen liquor and feces samples. Substance 5b also was present in large amounts in these samples.

In liver samples, 75 per cent of the activity was present as vitamins B_{12} and B_{12b}. Although Table IV indicates that 53 per cent of the activity in sheep kidney was due to Substance 5b, the proximity of Substances 5b and 6 in separations performed on kidney samples made accurate sectioning of the corresponding strips difficult, and this value is considered in question.

Radioautographs of the samples analyzed in the sheep experiment indicated that the specific activity (Co^{60}) of the vitamin B_{12}-active substances in this experiment were not nearly as high as the specific activities of the substances formed in earlier experiments *in vitro*. Nevertheless, Co^{60} was detected in Substances 2, 4, 5b, 6, and 7 in sheep feces. Samples of rumen liquor exhibited radioactivity in all of these except Substance 7 and in Substances 1 and 5a as well. Sheep liver contained radioactivity in Substances 4, 5a, 5b, and 6, while kidney samples exhibited radioactivity only in Substances 4, 5a, and 6. Since all of the vitamin B_{12}-active substances shown to exist here had Co^{60} activity in at least one of the samples, the failure to find Co^{60} in certain zones was probably due merely to the activity being too low to be detected efficiently by radioautography.

DISCUSSION

The results presented demonstrate the ability of rumen microorganisms to synthesize several substances having vitamin B_{12} activity for test organisms, but differing from vitamin B_{12} and vitamin B_{12b} in electrophoretic mobility. The identities of these substances have not been determined as yet. The results are, however, in accord with findings by other workers. In similar studies with rumen flora, Ford and Holdsworth (26) and Brown and Smith (27) isolated vitamin B_{12}-active substances which differed from cyanocobalamin in that the nucleotide base, 5,6-dimethylbenzimidazole, of cyanocobalamin was replaced by other purines; *e.g.*, adenine and 2-methyladenine. Similarly, Dion *et al.* (28, 29) showed that the purine portions of pseudovitamins B_{12} and B_{12d} were adenine and 2-methyladenine, respectively. Whether any of the substances demonstrated here are identical with those identified by other workers is not known at this time. However, since in all cases the bacteria producing the substances were from rumen origin, a relationship seems likely.

It is possible that one or more of the vitamin B_{12}-active substances are intermediates or precursors in the synthesis of the final vitamin B_{12} molecule. However, though there was active vitamin B_{12} synthesis for at least 72 hours by this system, no significant changes in the ratios of one substance to another occurred during this period of time.

Starch and other readily available sources of energy are known to change . the type of fermentation and microflora in rumen populations. This has been shown both by observation of the bacteria and in increased B vitamin

synthesis (30, 31). Its addition, in this system, did not produce any major changes in the proportions of the vitamin B_{12}-active substances. Total vitamin B_{12} activity synthesized was increased slightly, however. The addition of cobalt also increases the vitamin B_{12} production somewhat, but does not change the proportion of the various substances.

The vitamin B_{12} complex synthesized *in vivo* was very similar to or identical with that synthesized by rumen fermentations *in vitro*.

It is important to note that over half of the activity of rumen liquor may exist as substances other than vitamins B_{12} and B_{12b}. It is difficult to discuss the significance of the unidentified substances until both their chemical nature and biological activities have been fully determined. Coates *et al.* (15) stated that many of the substances were less active than vitamin B_{12} for chicks. This would suggest that these substances might not be of direct value to the ruminant animal. However, it is possible that they may serve in the metabolism of the rumen bacteria themselves.

It is of interest to state that Johnson (32) has observed that rat intestinal flora synthesize several vitamin B_{12}-active substances similar in electrophoretic mobility to those found in rumen liquor. When rats were fed a purified ration deficient in vitamin B_{12}, the vitamin B_{12} activity of the feces was increased by supplementing the ration with cobalt. Moreover, the liver and kidneys of the cobalt-supplemented group contained more vitamin B_{12} activity than those of the control group. This would indicate absorption from the large intestine, since the rats could not practice coprophagy.

The synthesis of vitamin B_{12} activity by rat intestinal flora was demonstrated earlier by Hartman *et al.* (33) and Davis and Chow (34). Also, Klosterman *et al.* (35) and Robison (36) showed that swine fed a vitamin B_{12}-deficient ration exhibited a growth response to cobalt supplementation. Similar effects of cobalt in chicks and rats were reported by Burns and Salmon (37). Thus, the microbial synthesis of vitamin B_{12} may be important in non-ruminants as well as in ruminants. This phase is being further investigated.

SUMMARY

1. Rumen microflora were grown both *in vitro* and *in vivo* in the presence of Co^{60}, and the vitamin B_{12}-active substances produced were separated by electrophoresis on filter paper.

2. Seven vitamin B_{12}-active substances having different electrophoretic mobilities were synthesized. Co^{60} was incorporated into all seven substances.

3. Two of the substances are vitamin B_{12} and vitamin B_{12b}. However, as much as 65 per cent of the total activity of the rumen liquor samples may be contributed by substances other than vitamins B_{12} and B_{12b}.

4. Four vitamin B_{12}-like substances occurred in sheep liver and kidney samples.

The authors are indebted to Merck and Company, Inc., through the courtesy of Dr. L. Michaud, for crystalline vitamin B_{12}.   Acknowledgment is also made to Dr. J. D. Sayre, Department of Agronomy of this Station, for helpful advice and for assistance in obtaining the isotope.

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TIGLYL COENZYME A AND α-METHYLACETOACETYL COENZYME A, INTERMEDIATES IN THE ENZYMATIC DEGRADATION OF ISOLEUCINE*
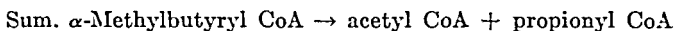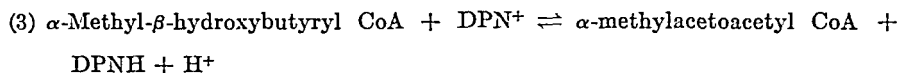
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Studies in this laboratory on the metabolic fate of α-methylbutyrate labeled in various positions with C^{14} have given strong support to the belief that this compound is an intermediate in isoleucine metabolism (1, 2). It was shown that, in liver tissue, α-methylbutyrate undergoes β oxidation on the longer carbon chain, followed by cleavage to produce "acetate" from carbons 3 and 4 and "propionate" from carbons 1 and 2 and the branched methyl carbon. These findings account satisfactorily for the known weakly ketogenic and slight, but definite, glycogenic properties of isoleucine, as discussed below. It has recently been found that the reactions under consideration proceed in soluble enzyme preparations of heart and liver only when α-methylbutyrate is present in the form of its CoA thiol ester,[1] thereby suggesting the occurrence of a metabolic pathway similar to that now widely recognized for straight chain fatty acid metabolism.

Preliminary results supporting the following reaction sequence have been reported briefly (3).
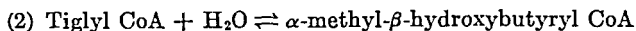
(1) α-Methylbutyryl CoA ⇌ tiglyl CoA (+2H)

(2) Tiglyl CoA + H_2O ⇌ α-methyl-β-hydroxybutyryl CoA

(3) α-Methyl-β-hydroxybutyryl CoA + DPN^+ ⇌ α-methylacetoacetyl CoA + DPNH + H^+

(4) α-Methylacetoacetyl CoA + CoA ⇌ acetyl CoA + propionyl CoA

Sum. α-Methylbutyryl CoA → acetyl CoA + propionyl CoA

The occurrence of Reactions 1 to 4 in pig heart and rat liver enzyme preparations is reported in the present paper. In addition, evidence is presented for the catalysis of Reaction 5 in heart extracts, due to the presence of CoA transferase.

(5) α-Methylacetoacetate + succinyl CoA ⇌ α-methylacetoacetyl CoA + succinate
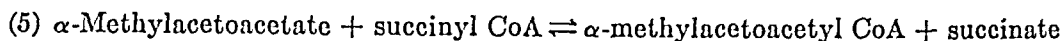
EXPERIMENTAL

Preparation of Heart and Liver Extracts—Rat livers or pig hearts obtained promptly after death of the animals were packed in ice and ground in a mechanical mincer. 20 gm. of minced tissue were homogenized with 20 ml. of 0.5 M potassium chloride at 0° in a Waring blendor for about 3 minutes; the resulting thick suspension was diluted with additional potassium chloride solution containing phosphate buffer and extracted at −7° with ethanol at a final concentration of 20 volumes per cent, according to procedures previously described in detail (4). The clear, red supernatant solution obtained upon centrifugation was dialyzed for 16 hours against 4 liters of potassium chloride, 0.04 M, containing phosphate buffer, pH 7.4, 0.025 M, and L-cysteine, 0.001 M. Typical liver and heart extracts prepared in this manner contained, respectively, 14.3 and 4.4 mg. of protein per ml. In some instances the extracts were brought to a final ammonium sulfate concentration of 0.37 gm. per ml. (0.7 saturation), and the precipitates obtained upon centrifugation were brought into solution and dialyzed as described earlier (4).

Hydration of Tiglyl CoA—With either the heart or liver extracts described, the hydration of tiglyl CoA (Reaction 2) was readily demonstrated by the spectrophotometric procedure of Stern and his associates.[2] This reaction most likely is brought about by crotonase (6) in the extracts employed, for a crystalline preparation of this enzyme (7) was found to hydrate tiglyl CoA. Since tiglic acid is known to be the cis isomer of α-methylcrotonic acid (8), it would appear that the observed hydration is an exception to the generalization of Wakil and Mahler (9) that this hydrase is specific for the trans isomers of α,β-unsaturated acyl derivatives.[3] Evidence for the identity of the hydration product was obtained by incubating tiglyl CoA with heart or liver extracts, converting the thiol esters present to the hydroxamic acids (11), and submitting the latter to chromatography in water-saturated butanol at 30°. The results of a typical experiment are presented in Table I (Experiment 1). In addition

[2] The hydration was measured by decrease in light absorption at 263 mμ, as described by Lynen and Ochoa (5). The details of this procedure were kindly furnished by Dr. J. R. Stern.

[3] It has recently been reported by other investigators that crystalline crotonase hydrates both *cis-* and *trans*-crotonyl CoA (10).

to tiglohydroxamic acid (R_F 0.72), a new compound was detected with an R_F of 0.54, corresponding to that of authentic α-methyl-β-hydroxy-butyrohydroxamic acid (R_F 0.56). As anticipated, only the former compound exhibited absorption when the paper chromatogram was viewed under ultraviolet light. Suitable controls indicated that tiglyl CoA is not hydrated in the absence of added enzyme under the conditions employed and that the enzyme preparation was free of any materials yielding hydroxamic acids.

Conversion of α-Methylbutyryl CoA to Tiglyl CoA and α-Methyl-β-hydroxybutyryl CoA—The dehydrogenation of α-methylbutyryl CoA (Reaction 1), predicted by analogy to the corresponding dehydrogenation of the CoA thiol

TABLE I

Chromatographic Identification of Hydroxamic Acids

Experiment No.	System	R_F values of hydroxamic acids
1a	Complete	0.72, 0.54
1b	No enzyme	0.72
1c	" tiglyl CoA	None
2a	Complete	0.71, 0.51
2b	No enzyme	0.79

In Experiment 1, the complete system contained 2.7 μmoles of tiglyl CoA, 2.5 μmoles of potassium Versenate, pH 7.4, and dialyzed rat liver extract (5.7 mg. of protein) in a final volume of 2.0 ml. Incubation, 10 minutes at 25°. In Experiment 2, the complete system contained 4 μmoles of α-methylbutyryl CoA, 50 μmoles of Tris buffer, pH 7.4, 1 mg. of cytochrome c, and dialyzed rat liver extract (2.8 mg. of protein) in a final volume of 0.70 ml. Incubation, 30 minutes at 38°.

esters of straight chain fatty acids (12), was demonstrated by the assay procedure of Green *et al.* (13). The following components were incubated at 38° in a final volume of 0.60 ml. in an evacuated Thunberg tube: Tris, pH 8.1, 50 μmoles; triphenyltetrazolium chloride, 3.6 μmoles; methylene blue, 0.2 μmole; α-methylbutyryl CoA, 2 μmoles; and dialyzed liver extract, 2.9 mg. of protein. The triphenyltetrazolium was found to be reduced at the rate of 0.2 μmole per hour. Tiglyl CoA, the expected product of the oxidation, is not distinguishable from α-methylbutyryl CoA by paper chromatographic methods currently in use, nor are the corresponding hydroxamates readily separated in this manner. By taking advantage of the hydration of tiglyl CoA by crotonase in the liver extract, however, it was possible to demonstrate chromatographically the formation of α-methyl-β-hydroxybutyryl CoA (hydroxamic acid, R_F 0.51) from α-methyl-butyryl CoA, as shown in Table I (Experiment 2).

Evidence for α-Methylacetoacetyl CoA As Intermediate—The postulated

DPN-dependent oxidation (Reaction 3) and cleavage of α-methylaceto-acetyl CoA to furnish acetyl CoA and propionyl CoA (Reaction 4) are analogous to enzymatic steps already recognized in straight chain fatty acid metabolism (14). When tiglyl CoA was incubated with oxalacetate and heart or liver preparations containing the citrate condensing enzyme (15), acetyl CoA liberation was readily demonstrated by citrate formation (Table II). The values recorded for Experiments 1 to 3 are corrected for a trace amount of citrate formed in control experiments in which tiglyl

TABLE II

Enzymatic Synthesis of Citrate from Tiglyl CoA and α-Methylacetoacetate

Experi-ment No	Enzyme preparation	Substrates added				Citrate formed
		Tiglyl CoA	Succinyl CoA	α-Methyl-aceto-acetate	DPN	
		μmoles	*μmoles*	*μmoles*	*μmoles*	*μmole*
1	Liver ammonium sulfate fraction (45.0 mg. protein)	2.0			3.0	0.41
2	" "	2.0				0.06
3	Heart extract (7.0 mg. protein)	2.0			3.0	0.26
4	Heart ammonium sulfate fraction (7.5 mg. protein)		4.0	50	3.0	0.48
5	" "		4.0		3.0	0.05

In addition to the substrates and enzyme preparations indicated, the test systems contained the following: Experiments 1 to 3, 500 μmoles of Tris buffer, pH 8.1, 20 μmoles of $MgCl_2$, 26 μmoles of cysteine, 125 μmoles of oxalacetate, and 0.25 μmole of CoA; volume, 3.6 ml.; incubation, 60 minutes at 38°. Experiments 4 and 5, 500 μmoles of Tris buffer, pH 8.1, 20 μmoles of $MgCl_2$, 13 μmoles of cysteine, 20 μmoles of l-malate, highly purified malic dehydrogenase (4 γ of protein), and crystalline condensing enzyme (16 γ of protein); volume, 2.5 ml.; incubation, 60 minutes at 38°. Citrate was estimated by the method of Natelson *et al.* (16).

CoA was omitted. It is apparent that citrate formation from tiglyl CoA in the liver preparation employed was dependent upon the addition of DPN.

The remaining experiments in Table II establish the formation of citrate from α-methylacetoacetate in a heart enzyme preparation supplemented with succinyl CoA and citrate condensing enzyme, along with malic dehydrogenase and malate as a source of oxalacetate. Only a trace of citrate was produced when α-methylacetoacetate was omitted from the reaction mixture (Experiment 5). These results suggest that the β-keto acid is activated by CoA transfer from succinyl CoA (Reaction 5) prior to the cleavage step liberating acetyl CoA (Reaction 4). This reaction is similar to the transfer of CoA from succinyl CoA to acetoacetate

known to occur in heart preparations (17, 18). Spectrophotometric evidence for the proposed CoA transfer, obtained with a highly purified preparation of heart CoA transferase,[4] is given in Fig. 1. The ascending portion of Curve A represents the formation of α-methylacetoacetyl CoA as indicated by increased absorption of light at 310 mμ (17) upon mixing the
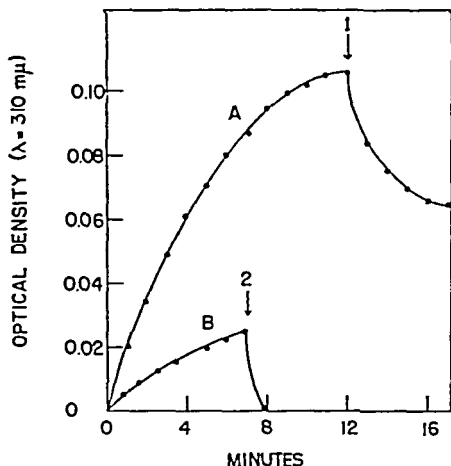

FIG. 1. Enzymatic synthesis of α-methylacetoacetyl CoA as a function of time. The ascending portion of Curve A represents α-methylacetoacetyl CoA formation on mixing CoA transferase (27 γ of protein) with 500 μmoles of Tris, pH 9.0, 50 μmoles of MgCl$_2$, 1 μmole of succinyl CoA, and 50 μmoles of α-methylacetoacetate in a cuvette in a final volume of 3.0 ml. At Arrow 1, heart 0 to 70 per cent ammonium sulfate fraction containing the acetoacetate condensing enzyme (thiolase) was added (1.5 mg. of protein). Sufficient amounts of free CoA to permit the occurrence of Reaction 4 were present in the reaction mixture. Curve B represents a similar experiment carried out at pH 8.1.

enzyme, succinyl CoA, and α-methylacetoacetate at pH 9.0. The addition (at Arrow 1) of a crude heart preparation containing the acetoacetate condensing enzyme (thiolase) resulted in disappearance of the product, apparently due to catalysis of α-methylacetoacetyl CoA cleavage according to Reaction 4. Similar results were obtained at pH 8.1, as indicated by Curve B, but the light absorption was much less than at the higher pH.

α-*Methylacetoacetate Formation in Heart and Liver Extracts*—If Reaction 5 is reversible, one would expect α-methylacetoacetyl CoA to yield α-methylacetoacetate in the presence of added succinate. This possibility was investigated in an experiment in which α-methylacetoacetyl CoA was generated enzymatically from tiglyl CoA (3 μmoles) in the presence of heart extract, DPN (4.5 μmoles), and potassium succinate (100 μmoles).

[4] Physical studies carried out by Dr. J. R. Stern demonstrated that the CoA transferase was homogeneous except for a trace of flavoprotein.

The β-keto acids formed were decarboxylated in the presence of aniline hydrochloride, and the 2,4-dinitrophenylhydrazones of the resulting ketones (19) were submitted to paper chromatography in methanol-saturated heptane by a modification of the procedure of Meigh (20). Two distinct yellow spots (R_F 0.65 and 0.47) were detected, corresponding to known methyl ethyl ketone dinitrophenylhydrazone (R_F 0.65) and acetone dinitrophenylhydrazone (R_F 0.46).[5] It is concluded from these results that α-methylacetoacetate was generated under the conditions employed. As anticipated, in a control experiment in which tiglyl CoA was omitted, the methyl ethyl ketone derivative could not be detected. In another control experiment in which succinate was omitted, however, the methyl ethyl ketone derivative was identified chromatographically. This unexpected finding may indicate that α-methylacetoacetyl CoA undergoes non-enzymatic hydrolysis and decarboxylation under the conditions employed to decarboxylate the free β-keto acids and form the hydrazones of the resulting ketones.

In similar experiments in which tiglyl CoA was incubated with rat liver extract, DPN, and cysteine, methyl ethyl ketone was detected chromatographically. Although these results support the conclusion that a β-keto acid (as the CoA thiol ester) is an intermediate in the metabolism of tiglyl CoA, they do not indicate whether α-methylacetoacetate is formed enzymatically from its CoA ester.

Conversion of α-Methylacetoacetate to Acetyl CoA and Propionyl CoA— Evidence is presented above for the liberation of acetyl CoA, measured by citrate formation, as a product of Reaction 4. The other product, propionyl CoA, was identified in an experiment in which tiglyl CoA was incubated with DPN, oxalacetate, and the dialyzed ammonium sulfate fraction of rat liver extract, and the resulting thiol esters were converted to the hydroxamic acids. Chromatography in water-saturated butanol established the presence of propionohydroxamic acid (R_F 0.61) in addition to the α-methyl-β-hydroxybutyrohydroxamic acid already described. Neither of these spots exhibited ultraviolet absorption, unlike tiglohydroxamic acid. Control experiments demonstrated that propionyl CoA was not further metabolized in the enzyme preparation employed and that no acetyl CoA accumulated in the presence of oxalacetate.

To establish the molar ratio of the products of the cleavage reaction, α-methylacetoacetyl CoA was generated in the heart enzyme system (supplemented with citrate condensing enzyme) under conditions leading to cleavage of this compound and conversion of the resulting acetyl CoA to

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[5] The acetone dinitrophenylhydrazone identified in this experiment arose from a trace of acetone present as an impurity in the commercial preparation of DPN employed. The CoA preparation used in these experiments contained no detectable carbonyl compounds.

citrate (Table III). Aliquots were taken for the determination of citrate and for chromatography of thiol esters at 4° in ethanol-acetate (21) on Whatman filter paper No. 3. The propionyl CoA (R_F 0.55) was eluted with water at 4° and assayed spectrophotometrically at 546 mμ by the nitroprusside reaction as described by Lynen (22), with propionyl CoA prepared from reduced CoA and propionic anhydride as a standard. The concentration of the chemically prepared propionyl CoA was in turn established by hydroxamic acid formation relative to an ethyl propionate standard. As may be seen in Table III, citrate was detected only when both succinyl CoA and α-methylacetoacetate were present in the reaction mixture. The ratio of acetyl CoA (citrate) to propionyl CoA was 1.26,

<div align="center">TABLE III</div>

Enzymatic Cleavage of α-Methylacetoacetate to Acetyl CoA and Propionyl CoA

System	Compound measured	Amount of compound
		μmoles
Complete	Propionyl CoA	0.88
"	Citrate	1.11
No succinyl CoA	"	0
" α-methylacetoacetate	"	0
Molar ratio, acetyl CoA (as citrate) to propionyl CoA. $\quad$ ..		1.26

The complete system contained 500 μmoles of Tris buffer, pH 8.1, 20 μmoles of magnesium chloride, 13 μmoles of cysteine, 3 μmoles of DPN, 200 μmoles of l-malate, 5 μmoles of succinyl CoA, 100 μmoles of α-methylacetoacetate, highly purified malic dehydrogenase (4 γ of protein), crystalline condensing enzyme (16 γ of protein), and dialyzed heart extract (8.4 mg. of protein); volume, 4.3 ml.; incubation, 60 minutes at 38°.

thereby establishing the formation of these products in almost equimolar amounts. A value closer to 1.0 would be obtained if allowance were made for the destruction of propionyl CoA under the incubation conditions employed (1 hour at 38°, pH 8.1). A control experiment established that about 37 per cent of a sample of known propionyl CoA disappeared under these conditions, but an exact correction cannot be applied to the calculated molar ratio in the absence of information on the rate at which propionyl CoA was released from α-methylacetoacetyl CoA during the experiment.

<div align="center">DISCUSSION</div>

In striking contrast to the strongly ketogenic nature of leucine, it appears from an examination of the available evidence that isoleucine and α-methylbutyrate are only weakly ketogenic (23–28) and that iso-

leucine has slight, but definite, glycogenic properties (29, 25, 30). The present study, establishing the formation of equimolar amounts of acetyl CoA and propionyl CoA from intermediates in isoleucine metabolism, satisfactorily accounts for these properties. The complete catabolism of a mole of the amino acid could under suitable conditions, as in the diabetic organism, lead to the formation of about 0.5 mole of glucose from propionate (31, 32) and 0.5 mole of ketone bodies from acetyl CoA.

The enzymatic Reactions 1 to 4 established in the present study account adequately for the isotopic results obtained earlier (1, 2), and the findings are in general agreement with the conclusion of Carter (33) that α-methyl fatty acids undergo β oxidation through the longer carbon chain only.

Whether the branched chain compounds produced from isoleucine serve any specific function other than as a source of energy in the metabolism of animals is unknown at the present time. It is interesting in this regard that the carbon skeleton of α-methylacetoacetate, for example, corresponds to that of isoprene, suggesting the possibility that isoleucine metabolites, like leucine metabolites (cf. (4)), may be precursors of complex branched molecules such as steroids. Since the condensation of acetyl CoA units to furnish acetoacetyl CoA is known to proceed readily, there is no reason to believe that the condensation of acetyl CoA and propionyl CoA to furnish α-methylacetoacetyl CoA (the reverse of Reaction 4) may not also occur in animal tissues. Such a mechanism would provide a simple means of obtaining branched chain compounds from substrates readily available from carbohydrate and lipide metabolism.

Methods

dl-α-Methylbutyric acid and tiglic acid were commercial products. The latter compound, purified by sublimation and distinguished from the trans isomer (angelic acid) by the melting point, 65–66° (7), was found to give only one spot (R_F 0.59) upon paper chromatography in 100:15:1 *n*-butanol-water-diethylamine (34). The ethyl ester of α-methyl-β-hydroxybutyric acid, synthesized according to Blaise and Herman (35), was converted to the corresponding hydroxamic acid under standard conditions and found to give a single component upon paper chromatography. α-Methylacetoacetate was made by alkaline hydrolysis of the ethyl ester, prepared according to Montagne (36); a sample of the β-keto acid was decarboxylated in the presence of aniline hydrochloride, and the 2,4-dinitrophenylhydrazone of the resulting methyl ethyl ketone was found to be chromatographically pure. Coenzyme A thiol esters of α-methylbutyric and tiglic acids were prepared by the general method of Wieland and Rueff (37), and succinyl CoA was made from succinic anhydride according to Simon and Shemin (38). The concentration of thiol esters prepared in this manner was esti-

mated on the basis of sulfhydryl disappearance, as determined by the method of Grunert and Phillips (39).

Malic dehydrogenase, crystalline crotonase, CoA transferase, and crystalline condensing enzyme were kindly furnished by Dr. J. R. Stern and Dr. S. Ochoa.

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SUMMARY

1. Evidence is presented for the occurrence of the following reactions in enzyme extracts of pig heart and rat liver: (a) the oxidation of α-methyl-butyryl CoA to tiglyl CoA; (b) the hydration of tiglyl CoA by crotonase to furnish α-methyl-β-hydroxybutyryl CoA; (c) the DPN-dependent oxidation of α-methyl-β-hydroxybutyryl CoA to α-methylacetoacetyl CoA; (d) the cleavage of α-methylacetoacetyl CoA to yield equimolar amounts of acetyl CoA and propionyl CoA.

2. Highly purified heart CoA transferase has been found to catalyze the following reaction: α-methylacetoacetyl CoA + succinate ⇌ α-methylaceto-acetate + succinyl CoA.

3. Under appropriate conditions, as in the diabetic organism, the complete catabolism of a mole of isoleucine according to the reactions established could give rise to about 0.5 mole of glucose (from propionyl CoA) and 0.5 mole of ketone bodies (from acetyl CoA), thereby accounting for the known glycogenic and weakly ketogenic properties of the amino acid.

4. It is suggested that the condensation of acetyl CoA and propionyl CoA to yield α-methylacetoacetyl CoA may provide a means of synthesizing branched chain compounds from simple straight chain substrates in animal tissues.

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METABOLISM OF THE ESSENTIAL FATTY ACIDS

II. THE METABOLISM OF STEARATE, OLEATE, AND LINOLEATE BY FAT-DEFICIENT AND NORMAL MICE*

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Although the symptoms of essential fatty acid deficiency have been extensively studied, the mechanisms by which these symptoms are produced are still obscure. Several groups have made observations suggesting investigations which might throw some light on this problem. For example, knowledge of the lipotropic effect of linoleic acid (1, 2) has resulted in the implication of this substance in the transport of fatty acids (3) and cholesterol (4), and several investigators (5–7) have shown that the fat-deficient state results in interference with oxidative phosphorylation. Perhaps the most revealing data on this subject appear in the work of Smedley-MacLean and Hume (8), who observed that rats on a low fat diet from weaning nevertheless had a higher percentage of body fat than did normal controls, even though the total body weight of the latter was considerably higher. When the fat-deficient animals received a linoleate supplement, however, body fat decreased and weight gain was resumed. From this evidence one may speculate that, although fat may be synthesized and deposited in fat deficiency, it cannot be used for the formation of new tissue.

In following this lead, we have investigated the metabolism of three typical 18-carbon fatty acids by fat-deficient and by normal mice. Stearate was used as a typical saturated acid, oleate as the main unsaturated acid of the animal body, and linoleate as a representative polyunsaturated fatty acid. In each case the rate of oxidation of the fatty acid was estimated from the respiratory CO_2. The incorporation of the C^{14}-labeled carboxyl carbon from the fed acid into other lipides was also studied.[1]

* This paper is based on work performed under contract No. AT-04-1-gen-12 between the Atomic Energy Commission and the University of California at Los Angeles.

[1] Recently Reinins and Turpeinen (9) have fed biosynthetically labeled linoleic acid to a normal and a fat-deficient rat and have checked the activity in the respiratory CO_2 and carcasses.

EXPERIMENTAL

Materials and Methods—Carboxy-labeled methyl stearate, methyl oleate, and methyl linoleate (10) were prepared by Dr. J. C. Nevenzel and Dr. D. R. Howton of this laboratory. Doses of the appropriate methyl ester diluted with corn oil to a total activity of 10^6 disintegrations per second[2] were administered orally[3] to fat-deficient[4] or normal mice. The animals were then placed in all-glass metabolism cages and the respiratory carbon dioxide was collected in sodium hydroxide solution hourly for 8 hours and at 10 and 24 hours. Barium carbonate was prepared from these samples, weighed for total respiratory CO_2, and checked for activity.[5] The animals were sacrificed at the end of each experiment, and the carcasses were frozen until used. Pooled carcasses from each group were extracted as described previously for rats (15). The total lipides were saponified, and the fatty acids thus obtained were separated into saturated and unsaturated fractions by the lead-salt technique (16). The total unsaturated fatty acids were brominated in the usual manner (15, 17) and separated into crude "octabromide," "hexabromide," and "tetrabromide" fractions. The crude "octabromide" fraction was partially purified by washing with hot benzene and ether. The "tetrabromide" fraction was washed thoroughly with petroleum ether (b.p. 60–70°) and, when possible, crystallized from ethylene chloride. In one case (oleate-fed normal mice) the tetrabromostearic acid was recrystallized to constant activity from ethylene chloride and acetone. The non-saponifiable fractions were weighed and then crystallized from ethanol, acetic acid,

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[2] In three cases (see Table II) a much higher dose was inadvertently administered. Respiratory CO_2 was not measured with these animals, and the carcasses were worked up separately.

[3] It was recognized that oral administration of the tagged material would result in a delay in utilization because of absorption from the gut. However, data were available in this laboratory which would enable this delay to be discounted if necessary (11), and there is evidence in the literature that intravenous injection of a fatty emulsion may not duplicate normal conditions, since the rate of oxidation is markedly influenced by particle size (12).

[4] Male mice had been on a fat-free diet 3 months from weaning. These animals and the experimental diets used are described in a previous paper (13). The mean weight of the eleven fat-deficient mice used in the experiment was 22.5 gm. as compared with 27.5 gm. for the thirteen normal animals.

[5] Counting was performed with a Nuclear Instrument and Chemical Corporation flow counter or a thin end window counter on samples prepared according to standard procedures. Lipides were plated directly on 1 inch aluminum planchets with lens paper (14). Some of these values were checked against $BaCO_3$ derived from duplicate samples. $BaCO_3$ was plated in the usual manner on 1 inch aluminum planchets. All counts were corrected for self-absorption by using curves determined for $BaCO_3$ or fatty acids. $BaCO_3$ counts were corrected for back-scattering to obtain values comparable to those from fatty acids.

and acetonitrile to constant activity and correct melting point for choles-
terol.   All fractions were weighed and counted.

TABLE I

Excretion of C^{14} by Normal and Fat-Deficient Mice Fed Carboxyl-Labeled Esters

Ester fed	No. and state of mice	Mean weight	24 hr. respiratory CO_2	Urine	Total ± s.d.
		gm.			
Stearate	3 normal	27.0	29.1	0.28	29.4 ± 8.8
	3 fat-deficient	23.6	50.6	0.04	50.7 ± 2.3
Oleate	3 normal	30.6	57.8	0.83	58.6 ± 1.7
	4 fat-deficient	23.0	48.7	0.31	49.1 ± 5.1
Linoleate	7 normal	26.1	38.4	0.21	38.6 ± 3.9
	3 fat-deficient	20.6	57.5	0.43	57.9 ± 4.6
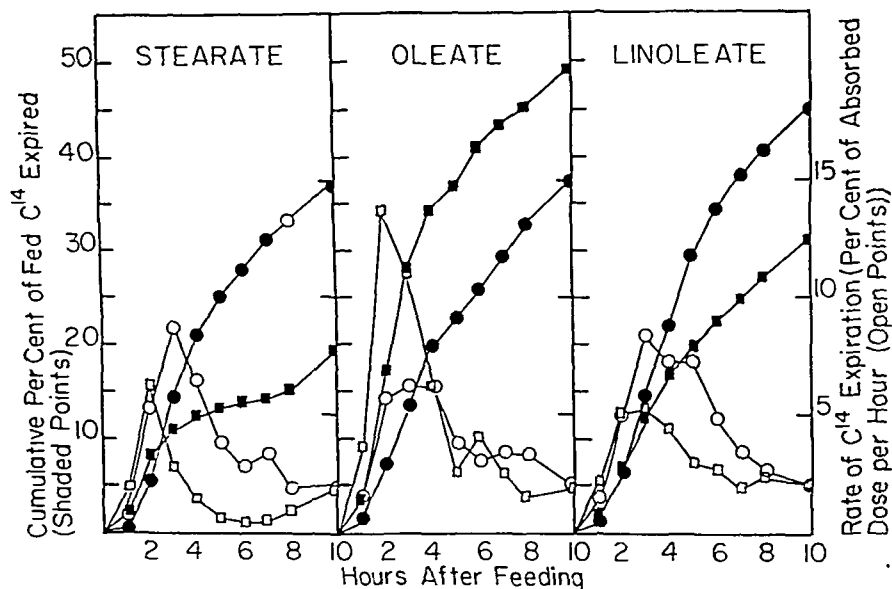
FIG. 1. Recovery of C^{14} in expired CO_2 after feeding carboxy-labeled stearate,
oleate, and linoleate to normal (squares) and fat-deficient (circles) mice.

Results

Data on the total excretion of C^{14} (Table I) indicate that stearate and
linoleate were oxidized to a significantly greater extent by fat-deficient
than by normal mice, while for oleate the reverse was true.   However,
despite the greater rate of oxidation by the fat-deficient mice (Fig. 1),
the peak of activity in the respiratory carbon dioxide was significantly

later than for normal animals. It is possible that this finding stems from the fact that with these animals, apparently nervous and irritable, the fed oil was retained for longer periods in the stomach. This condition has been observed with other mice in what appeared to be a highly nervous state (11).

TABLE II

Incorporation of Carboxyl Carbon from Fed Fatty Acids into Lipides of Normal and Fat-Deficient Mice

No. and state of mice	Total activity administered, disintegrations per sec. $\times 10^6$	Specific activity (disintegrations per sec. per mg.) of							
		Total lipide	Choles-terol*	Total acids	Satu-rated acids	Unsatu-rated acids	Tetrabro-mide fraction†	Hexabro-mide fraction†	Octabro-mide fraction†
Stearate									
4 normal	5	115	55	99	247	48	33	14	3
		(10)	(0.27)‡	(8)	(1.88)	(2)	(0.006)	(0.060)	(0.016)
3 fat-deficient	3	76	17	85	119	70	71		
		(8)	(0.25)‡	(8)	(2.86)	(1.72)	(0.0004)		
Oleate									
3 normal	3	81	15	100	72	99	13	32	12
		(7.1)	(0.225)‡	(6.3)	(1.6)	(3.5)	(0.260)	(0.150)	(0.160)
4 fat-deficient	4	69	33	67	73	81	69	5	7
		(17.7)	(0.20)‡	(13.8)	(6.2)	(3.4)	(0.052)	(0.002)	(0.071)
Linoleate									
3 normal	15	134	181	149	71	301	475		208
		(10)	(0.225)‡	(7.1)	(2.39)	(3.60)	(0.303)		(0.110)
4 fat-deficient	3	117	12	149	113	521	730		690
		(10)	(0.391)‡	(6.1)	(3.19)	(0.49)	(0.0035)		(0.0022)

The figures in parentheses represent the weight of the fractions in gm.

* Recrystallized to constant activity.

† Calculated on the basis of bromine-free acids.

‡ Non-saponifiable.

Finally, it is of interest to note that there is apparently no sparing of methyl linoleate which, especially in the fat-deficient animals, was oxidized at as great a rate as were the non-essential acids. It is possible that only those essential fatty acids in the important end-organs are spared by some mechanism which does not operate in the case of newly ingested acids.

In Table II are shown the weights and activities of lipides derived from the different groups of mice. The activity of the cholesterol (as compared

to that of total fat) appears to bear an inverse relationship to that of the total respiratory CO_2 (Table I). Apparently the condition of fat deficiency does not seem to hinder either the hydrogenation of unsaturated or the dehydrogenation of saturated acids, as shown by the activity of the unsaturated acids from the stearate-fed animals and of the saturated acids from those in other groups.

Some conclusions may be drawn from the activities of the polybromides when these were obtained in large enough quantities for adequate purification. The activity of the octabromide derived from the linoleate-fed normal mice could not be removed by solvent extraction and may indicate a conversion of linoleate into arachidonate. In the case of the oleate-fed normal mice, the tetrabromostearic acid was crystallized nine times from acetone or ethylene chloride. The activity was constant after the fifth crystallization. However, this activity is not to be taken as proof that the mouse can convert oleic to linoleic acid, since it was found that methyl linoleate was present as an impurity in the fed methyl oleate in amounts large enough to account for the observed activity. A discussion of these latter results will appear in a subsequent paper in which the metabolic pathway of linoleic acid is considered.

DISCUSSION

From the results reported above, several statements can be made with some assurance. Essential fatty acid deficiency certainly does not decrease the ability of the animal to catabolize fat; in fact, in two cases, this ability was evidently enhanced. The discrepancy in the case of oleic acid cannot be explained at the present time; should future experiment show this case to be unique, essential fatty acid deficiency may prove to be characterized generally by an increase in catabolic at the expense of anabolic reactions. The high metabolic rate of fat-deficient animals was noted by Wesson and Burr (18) in their early studies and has since been confirmed by several groups (19, 20). It is of interest to note that the catabolism of fatty acid and the incorporation of tracer into cholesterol apparently bear an inverse relationship. However, an experiment designed to test the capacity of the fat-deficient mouse to synthesize cholesterol from acetate indicated no disability of this type.[6]

The fact that linoleate was oxidized at a high rate even by fat-deficient mice seems inconsistent with its essential nature, except for the possibility that conservation of the essential fatty acids may be a characteristic of certain organs although not taking place to so great an extent in others

[6] Fat-deficient and normal mice received intraperitoneal injections of carboxylabeled sodium acetate. No significant difference was found in the C^{14} content of the cholesterol derived from these two groups of animals.

(21). The data on the relative activities of the saturated and unsaturated fatty acids appear to have no unusual features. The fat-deficient animal is apparently not limited in its ability to saturate or unsaturate fatty acids. It is evident that linoleic acid is not so rapidly converted to saturated acids as is oleic acid, although this reaction apparently does take place readily. The activities of the various polybromide fractions may be interpreted as follows: In the case of the linoleate-fed animals, a true conversion of diene to tetraene and other polyenes may take place.[7] In the other cases, direct transformation of the fed acid to linoleate and more highly unsaturated acids is extremely unlikely. Activity in these fractions is present either because of a slight impurity in the fed acid (as in the case of oleic acid), or by addition to inactive linoleic acid of acetyl fragments formed by breakdown of the fed acid (15), or, in some cases, because of the difficulty of purifying extremely small amounts of these substances.

Data obtained in these experiments have indicated that further research in this field, the influence of the essential fatty acids on lipide metabolism, may provide answers to some of the questions concerning the nature and action of these substances.

SUMMARY

Normal and fat-deficient mice were given orally carboxy-labeled methyl stearate, oleate, or linoleate. The C^{14} content of the respiratory carbon dioxide and various body lipids was determined. Fat-deficient mice metabolized stearate and linoleate at a significantly greater rate than did normal mice and oleate at a lower rate. The incorporation of C^{14} into cholesterol seemed to bear an inverse relationship to that in total respiratory CO_2. No evidence could be found for the conversion of oleate to linoleate *in vivo*, but linoleate may be converted to arachidonate.

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[7] This study forms the basis of a separate paper to be issued in the near future.

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CONVERSION OF TESTOSTERONE TO ESTROGENS IN CASTRATED, ADRENALECTOMIZED HUMAN FEMALES*

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To explain certain clinical observations, such as feminization in pubescent boys and men with testicular tumors, it has been suggested that androgens may be metabolized to estrogens. Experiments in both animals and man support this theory. As early as 1936, Steinach and coworkers reported an increased urinary excretion of biologically active estrogens after the administration of androgens to normal and castrated rats (1) and normal men (2). These original observations were confirmed by other investigators and extended to other animals, eunuchoid men, and castrated women (3–6).

Dorfman and Hamilton demonstrated that this urinary estrogenic material was phenolic, indicating that true estrogens, rather than neutral steroid metabolites with estrogenic properties, were probably involved (6). Nathanson *et al.* reported the identification of estrone, estradiol-17β, and estriol in urines of ovariectomized women with breast cancer on androgen therapy (7). Identification was based on counter-current distribution, but details of the criteria used for characterization of the individual estrogens were not described.

In all these experiments, the urinary estrogens could have originated from the adrenal cortex rather than from metabolism of administered androgens. Evidence is presented in this report for the conversion of testosterone to estrogens in the absence of both ovaries and adrenals. Estrone and estradiol-17β were identified in the urine of castrated, adrenalectomized women with breast cancer following testosterone administration. The conversion of testosterone to estriol could not be established.

EXPERIMENTAL

Clinical Material—The first patient (K. H.) was a 33 year-old woman who had a radical mastectomy for breast cancer 7 years prior to this study. The breast operation was followed by a prophylactic bilateral oophorectomy. With the appearance of generalized metastases, a bilateral adrenalectomy was performed as a therapeutic procedure.

14 months after adrenalectomy, a control urine sample was collected for 3 days, after which the patient was started on 200 mg. of testosterone propionate intramuscularly daily. A 3 day urine sample was collected on the 8th to the 10th day of testosterone therapy. The patient had to be maintained on 75 mg. of cortisone acetate daily during the entire experiment in order to prevent adrenal insufficiency.

The second patient (M. L.) was a 53 year-old woman who developed breast cancer 5 years prior to this experiment. Generalized osseous metastases occurred $2\frac{1}{2}$ years later and a bilateral oophorectomy was performed, with good therapeutic results. Upon relapse, she underwent bilateral adrenalectomy.

At the beginning of this experiment, the patient was being treated with 100 mg. of testosterone propionate intramuscularly daily. A 24 hour urine sample was collected while she was on testosterone therapy. The testosterone was then discontinued for 1 month, after which a 24 hour control urine sample was collected. The patient received 75 mg. of cortisone daily during both the control and experimental periods.

Hydrolysis and Extraction—The urines were hydrolyzed by incubating them for 5 days at 37° with 300 units of β-glucuronidase[1] per ml. of urine, as recommended by Gallagher *et al.* (8). Acid hydrolyses were not done because of the relatively low proportion of estrogen conjugates other than glucuronidates in human urine (8). The hydrolyzed urines were extracted continuously with ether for 48 hours. The phenolic fractions were prepared from the ether extracts, as described by Engel (9), and partitioned between 70 per cent ethanol and petroleum ether prior to counter-current distribution.

Counter-Current Distribution—The three major urinary estrogens, estrone, estradiol-17β, and estriol, were separated out of the phenolic fractions and identified by counter-current distribution methods developed by Craig and Craig (10) and adapted for the analysis of estrogens by Engel (9). A 100 tube counter-current instrument designed by Craig *et al.* (11) was used. The solvent systems and the partition coefficients for estrone, estradiol-17β, and estriol are given in Table I. The theoretical distribution curves and amounts of estrone, estradiol-17β, and estriol were

[1] Ketodase (Warner-Chilcott).

calculated from the experimental data as described by Craig and Craig (10).

The degree of deviation of the experimental distribution curve from the theoretical was used as an index of the completeness of separation of the estrogens from other fluorescent materials. Separation was considered complete and identity established when the experimental distribution curve agreed with the standard theoretical distribution curve within the limits of error of the analytical method. In order to satisfy these criteria for the isolation and identification of estrogens, it was necessary to use multiple distributions with different solvent systems.

TABLE I

Solvent Systems Used in Counter-Current Distribution and Partition Coefficients for Estrone, Estradiol-17β, and Estriol

Solvent System No.	Solvent	K (partition coefficient)*		
		Estrone	Estradiol-17β	Estriol
1	50% CH₃OH-CCl₄	0.33	2.07	16.0
2	70% CH₃OH-40% CHCl₃, 60% CCl₄	0.21	1.02	5.45
3	40% EtOAc, 60% C₆H₁₂-60% EtOH	0.96		
4	50% EtOAc, 50% C₆H₁₂-33% "			1.20
5	60% CH₃OH-90% CHCl₃, 10% EtOAc			0.53

* K is the concentration of the solute in the upper phase divided by the concentration in the lower phase. The K values were determined by counter-current distribution.

Photofluorometric Analysis—Estrone, estradiol-17β, and estriol were measured quantitatively by photofluorometry, as described by Bates and Cohen (12) and modified by Engel (9). Samples for analysis were dissolved in 0.2 ml. of alcoholic toluene (1 part ethanol to 19 parts toluene); 1 ml. of 90 per cent sulfuric acid was added and the mixture was heated in a water bath at 80° for 15 minutes. After cooling, 6 ml. of 65 per cent sulfuric acid were added. The fluorescence was read against the proper estrogen standard in a Farrand photofluorometer, model No. A. Corning glass filters (Nos. 3389 and 5113) with a maximal transmission at 436 mμ were used as lamp filters. Combinations of an interference filter (485 mμ) and Corning glass filters (Nos. 3387 and 4308) were used as photocell filters.

It was necessary to distill the absolute ethanol (U. S. Industrial grade), toluene (Baker, c.p.), and sulfuric acid (Grasselli-du Pont) in order to reduce the blank fluorescence to an acceptable level.

Paper Chromatography—Paper partition chromatography, as developed by Zaffaroni (13) and adapted for estrogens by Axelrod (14), was used as an additional criterion for the identification of estrogens. Paper chromatograms for estrone were developed in either toluene-propylene glycol or cyclohexene-formamide solvent systems. For estradiol-17β and estriol, a methylene dichloride-formamide system was used. Appropriate estrogen reference standards were run simultaneously with unknowns. Phenolic compounds were visualized on the chromatograms by testing with an aqueous solution of 1 per cent ferric chloride and 1 per cent potassium ferricyanide (14).

Results

Conversion of Testosterone to Estrogens (Patient K. H.)—The control urinary phenolic fraction and that obtained while patient K. H. was on
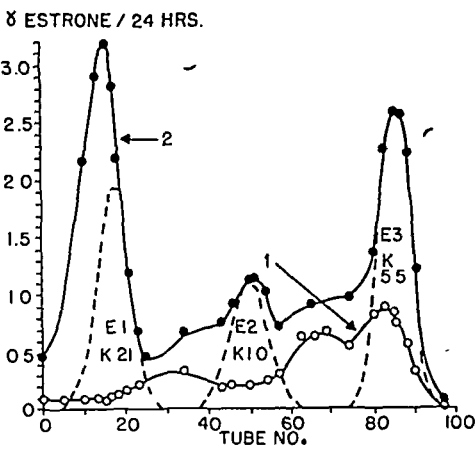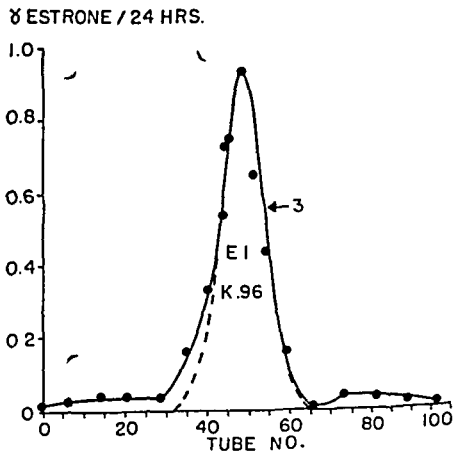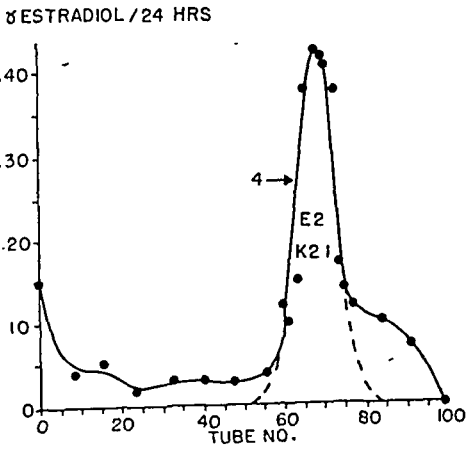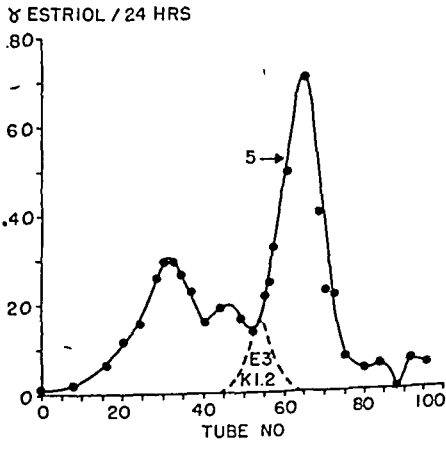

FIG. 1, *A*

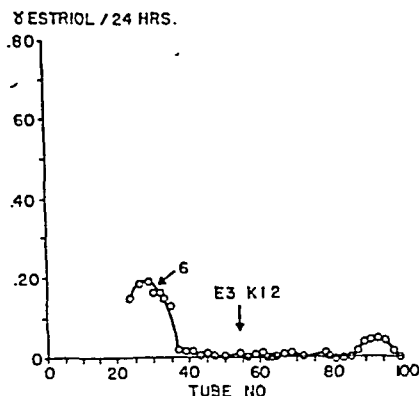FIG. 1, *B*

FIG. 1, *C*

FIG. 1, *D*

FIG. 1, *E*

FIG. 1. Counter-current analyses for urinary estrogens (patient K. H.). O, control; ●, on 200 mg. of testosterone propionate daily; broken line, theoreticals for estrone (E1), estradiol-17β (E2), and estriol (E3) with partition coefficients (*K*). *A*, original distributions during control (Curve 1) and on testosterone propionate (Curve 2). *B*, Curve 3 from redistribution of tubes 15 to 19 (Curve 2) in Solvent System 2. *C*, Curve 4 from redistribution of tubes 49 to 52 (Curve 2) in Solvent System 1. *D*, Curve 5 from redistribution of tubes 83 to 87 (Curve 2) in Solvent System 4. *E*, Curve 6 from redistribution of tubes 83 to 87 (Curve 1) in Solvent System 4.

testosterone were first distributed in Solvent System 2 (Table I). The distribution curves for both are shown in Fig. 1, *A* (Curves 1 and 2) along with the theoretical curves for estrone, estradiol-17β, and estriol.

The control distribution curve (No. 1) revealed that neither estrone nor estradiol-17β was excreted in detectable amounts before testosterone was administered. Curve 1 had the same maximum (tube 85) as the theoretical curve for estriol, but the marked deviation of the experimental from the theoretical indicated that this fluorescent material was not homogeneous, and identification of estriol could not be made on the basis of this distribution. Therefore, the contents of tubes 83 to 87 of Curve 1 were combined and redistributed for estriol in Solvent System 4 (Curve 6, Fig. 1, *E*). No estriol was found.

With testosterone administration, the total amount of fluorescent material in the phenolic fraction increased considerably. Three non-homogeneous fluorescent fractions separated out during the first counter-current distribution (Fig. 1, *A*, Curve 2). The distribution curve for the least polar material had a maximum at tube 15, as compared with a maximum at tube 18 for estrone. The distribution curve of the second fraction had the same maximum as estradiol-17β. The third, most highly polar, fraction had the same maximum as estriol.

In each case there was considerable discrepancy between the experi-

mental and theoretical curves, indicating that mixtures of fluorescent phenolic compounds were involved. In order to separate these mixtures, thereby permitting identification of individual estrogen components, it was necessary to combine the peak tubes for estrone, estradiol-17β, and estriol, respectively, and redistribute the material in different solvent systems. Accordingly, tubes 15 to 19 from Curve 2 were combined and redistributed for estrone in Solvent System 3, tubes 49 to 52 (Curve 2) were redistributed for estradiol-17β in Solvent System 1, and tubes 83 to 87 (Curve 2) were redistributed in Solvent System 4 for estriol.
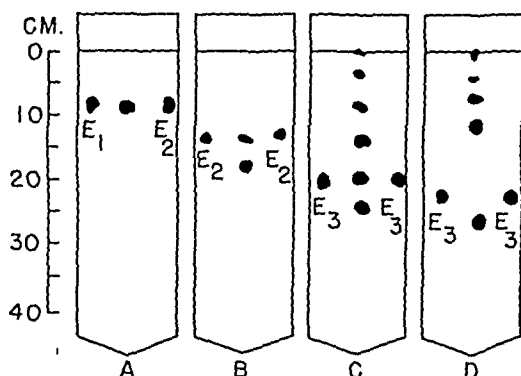
FIG. 2. Diagrams of paper chromatograms in the chromatographic analysis for urinary estrogens (patient K. H.). Reference standards are estrone (E_1), estradiol-17β (E_2), and estriol (E_3). Chromatogram A, tubes 15 to 19 from counter-current distribution Curve 2, developed in cyclohexene-formamide for 28 hours. Chromatogram B, tubes 65 to 71 (Curve 4), methylene dichloride-formamide for 6 hours. Chromatogram C, tubes 57 to 63 (Curve 5), methylene dichloride-formamide for 94 hours. Chromatogram D, tubes 57 to 63 (Curve 6), methylene dichloride-formamide for 99 hours.

Identification of Estrone (Patient K. H.)—The experimental curve obtained upon redistribution of tubes 15 to 19 (Curve 2) for estrone coincided with the theoretical curve for estrone, except for the base of the ascending limb of the curve (Curve 3, Fig. 1, *B*). The total 24 hour excretion of estrone, while the patient was on 200 mg. of testosterone propionate daily, was calculated to be 24.1 γ.

Additional evidence for the existence of estrone was obtained by paper chromatography. 10 per cent of the material in the pooled tubes (Nos. 15 to 19 from Curve 2) was applied to a paper chromatogram impregnated with formamide as the stationary phase and developed for 28 hours with cyclohexene saturated with formamide. With the ferric chloride-ferricyanide reagent previously described, a single phenolic compound was detected 10 cm. from the starting line (Chromatogram A, Fig. 2). The estrone reference standards, which were run simultaneously with the unknown, had moved the same distance, identifying the materi~~~ ~~ ~~trone

Identification of Estradiol-17β (Patient K. H.)—Upon redistribution of tubes 49 to 52 from Curve 2, two fluorescent fractions that were not completely separated were obtained (Curve 4, Fig. 1, C). The distribution curve for the less polar fraction agreed quite well with the theoretical curve for estradiol-17β, except for the base of the descending limb of the curve where there was incomplete separation from a slightly more polar fluorescent material. The total amount of estradiol-17β excreted, while the patient was on 200 mg. of testosterone propionate daily, was calculated to be 16.3 γ per 24 hours.

This identification by counter-current distribution was corroborated by paper chromatography. The contents of tubes 65 to 71 from Curve 4 were placed on a paper chromatogram with estradiol-17β reference standards. When the chromatogram was developed in a methylene dichloride-formamide system for 6 hours, two phenolic fractions separated out (Chromatogram B, Fig. 2). One had moved 14.8 cm. from the starting line, the same distance as the estradiol-17β standards. The other phenol was slightly less polar and had moved 19.0 cm. from the starting line.

Analysis for Estriol (Patient K. H.)—When the contents of tubes 83 to 87 of Curve 2 were redistributed for estriol, three non-homogeneous fluorescent phenolic fractions were partially separated (Curve 5, Fig. 1, D). Because the theoretical curve for estriol did not agree with the experimental curve, estriol could not be identified in this mixture. A comparison of Curve 5 with Curve 6 (control) suggests that some of the unidentified fluorescent phenolic material might have arisen from the administered testosterone.

Tubes 57 to 63 from Curve 5 were analyzed further for estriol by means of paper chromatography. The chromatogram was developed in a methylene dichloride-formamide system for 94 hours. Six distinct phenolic compounds separated out (Chromatogram C, Fig. 2). One compound had the same mobility as the estriol reference standards, another was slightly less polar than estriol, and the other four phenols were more polar than estriol.

Even though one of the phenolic compounds had the same mobility as estriol, it was not considered possible to identify estriol in this mixture of phenols with any degree of confidence because of the number of compounds so closely related to estriol in solubility characteristics, even after two successive counter-current distributions.

Similar unidentified phenols were demonstrated by paper chromatography in the control urine (Chromatogram D, Fig. 2).

Conversion of Testosterone to Estrogens (Patient M. L.)—The conversion of testosterone to estrogens in patient K. H. was confirmed in a second oophorectomized, adrenalectomized patient (M. L.). The phenolic

fractions from both the control urine and that collected while the patient was on testosterone propionate were prepared as described previously. The initial counter-current distribution of the phenolic fraction was carried out in Solvent System 1 with twenty-four transfers. The distribution curves obtained are shown in Fig. 3, A (Curves 7 and 8).

There were no detectable amounts of estrone or estradiol-17β in the initial distribution of the control urine, but estriol could not be ruled out. When tubes 22 to 24 of Curve 7 (control) were redistributed, as shown in Curve 12 of Fig. 3, E, estriol still could not be identified or definitely excluded.

Identification of Estrone (Patient M. L.)—To ascertain whether estrone was excreted in the urine while the patient was on testosterone, it was necessary to redistribute tubes 5 to 8 of Curve 8 (Fig. 3, A). Upon redistribution, Curve 9 (Fig. 3, B) was obtained, which agreed well with the
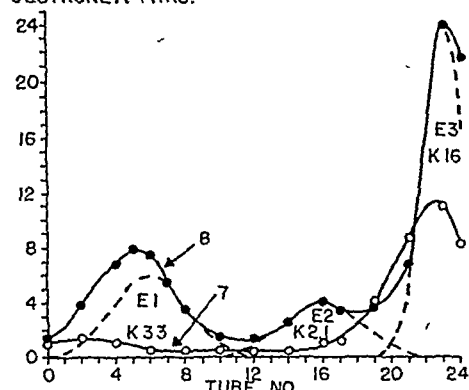
FIG. 3, A
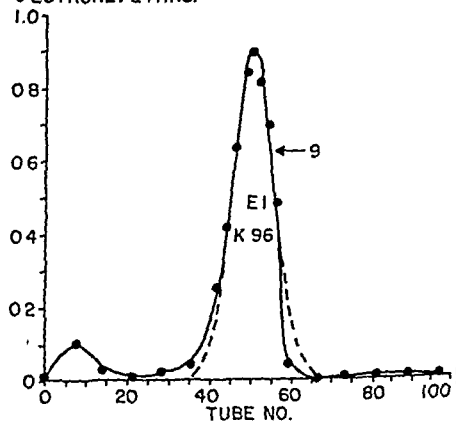
FIG. 3, B
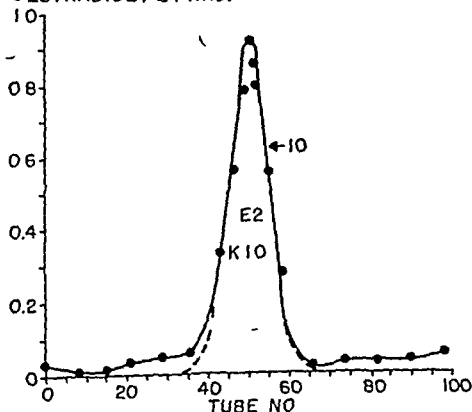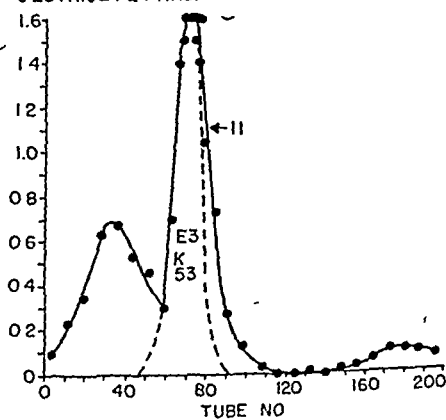
FIG. 3, C

FIG. 3, D
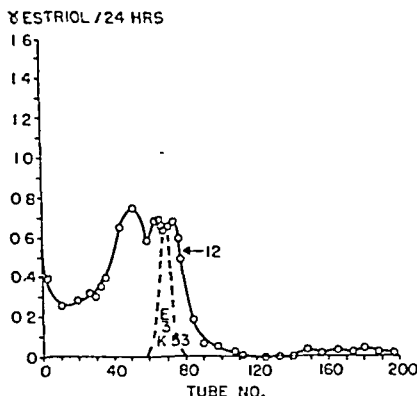
FIG. 3, *E*

FIG. 3. Counter-current analyses for urinary estrogens (patient M. L.). O, control; ●, on 50 mg. of testosterone propionate daily; broken line, theoreticals for estrone (E1), estradiol-17β (E2), and estriol (E3) with partition coefficients (*K*). *A*, original distributions during control (Curve 7) and on testosterone propionate (Curve 8). *B*, Curve 9 from redistribution of tubes 5 to 8 (Curve 8) in Solvent System 3. *C*, Curve 10 from redistribution of tubes 16 to 18 (Curve 8) in Solvent System 2. *D*, Curve 11 from redistribution of tubes 22 to 24 (Curve 8) in Solvent System 5. *E*, Curve 12 from redistribution of tubes 22 to 24 (Curve 7) in Solvent System 5.

theoretical curve for estrone. The total amount of estrone, calculated from the counter-current data, was 17.2 γ per 24 hours.

Estrone was also identified by means of paper partition chromatography. Tubes 48 to 52 from distribution Curve 9 were combined and run in a toluene-propylene glycol system for 20 hours along with estrone standards. A single phenolic fraction, having the same mobility as estrone, was identified 26 cm. from the starting line.

Identification of Estradiol-17β (Patient M. L.)—In a similar manner, estradiol-17β was identified in the phenolic fraction obtained while the patient was on testosterone. When tubes 16 to 18 of the initial distribution curve (No. 8) were redistributed, the experimental curve coincided with the theoretical curve for estradiol-17β (Curve 10, Fig. 3, *C*). The calculated amount of estradiol-17β was 25.5 γ per 24 hours.

Estradiol-17β was also identified in tubes 48 to 52 of Curve 10 by paper chromatography. The paper chromatogram with estradiol-17β reference standards was developed in a methylene dichloride-formamide system for 6 hours. In addition to estradiol-17β, a second phenolic compound, that was slightly less polar than estradiol-17β, separated out.

Analysis for Estriol (Patient M. L.)—Upon redistribution of tubes 22 to 24 from Curve 8 for estriol, two mixtures of fluorescent phenols were partially separated (Curve 11, Fig. 3, *D*). Although the maximum of the

experimental curve agreed with that for estriol, it could not be ascertained whether estriol was present in this mixture because of the failure of complete resolution of the component fluorescent phenols. Just as with patient K. H., these phenols could be separated by paper chromatography, but estriol could not be positively identified because of the number of phenols so closely related to estriol.

SUMMARY

When testosterone propionate was administered to two oophorectomized, adrenalectomized women with metastatic breast cancer, estrone and estradiol-17β were identified in the urine by the criteria of counter-current distribution and paper chromatography. Neither could be identified in the control urines when the patients received no testosterone. Inasmuch as both patients were castrated and adrenalectomized, it was concluded that the estrone and estradiol-17β originated from metabolism of the administered testosterone.

Many other unidentified phenols were demonstrated in the urine during testosterone therapy. It could not be established whether or not testosterone was converted to estriol, because of the number of phenolic compounds with solubility characteristics similar to those of estriol.

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TISSUE COENZYME A IN NORMAL AND INDUCED STATES OF GROWTH

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Results of studies on pantothenic acid-deficient rats stimulated with anterior pituitary growth hormone (1–3) suggest involvement of pantothenic acid, or its metabolically active form, coenzyme A (4), in the mechanism by which growth hormone stimulates the growth process. Lotspeich (1), for example, describes the rapid production of symptoms of acute pantothenic acid deficiency as a sequel to the administration of growth hormone in adult rats maintained on a pantothenic acid-deficient diet. Beare, Beaton, and McHenry (2) note that pantothenic acid-deficient rats fail to respond anabolically to growth hormone, while Hazelwood, Bennett, and Nelson (3) have recently reported a reduction in the nitrogen-retaining effect of growth hormone in normal and adrenalectomized pantothenic acid-deficient rats.

These observations, and those of Greenbaum (5) and Greenbaum and McLean (6, 7) on adult rats, emphasizing the effects of growth hormone on fat metabolism, in which coenzyme A is known to be intimately involved, have prompted us to study coenzyme A concentrations in liver and kidney tissue obtained from rapidly growing immature normal rats, from immature rats in which the growth process has been arrested by hypophysectomy, from normal adult rats, and from animals in the last two groups in which the growth process has been stimulated with growth hormone.

EXPERIMENTAL

Animals—Female rats of the Sprague-Dawley strain, both normal and hypophysectomized, were used in all experiments. The hypophysectomized rats were 28 to 30 days old at the time of operation.

Diet—All animals were housed in separate cages and were maintained on a diet containing 67.5 per cent ground whole wheat, 15 per cent casein, 10 per cent whole milk powder, 0.75 per cent sodium chloride, 1.5 per cent calcium carbonate, and 5.25 per cent corn oil (8). The diet was supplemented with fresh lettuce and haliver oil.

Methods—In general, body weight and food intake were determined

daily for each rat for a period of sufficient duration to establish whether the animals were growing or in an arrested state of the growth process. The animals were then grouped and observations continued for a period of 10 days. During this period nitrogen storage and gain in body weight were induced in a group of the hypophysectomized rats and in a group of the normal adult animals by administering intraperitoneally a lyophilized preparation of purified growth hormone[1] dissolved in 0.9 per cent saline. The hormone preparation was administered to the hypophysectomized rats in doses of 50 γ per day for 5 days and 100 γ per day for a subsequent 5 day period. In the experiments in which nitrogen storage was induced in normal adult rats, the hormone was administered in doses of 500 γ per day for the 10 day period. Upon termination of the 10 day period of hormonal stimulation, the treated rats and the untreated controls, both normal and hypophysectomized, were sacrificed by stunning and exsanguination. Liver and kidney were quickly excised, weighed, frozen on dry ice, and stored at $-15°$ to $-20°$ until convenient for coenzyme A determination. Coenzyme A concentrations in tissue were determined by the sulfanilamide acetylation procedure of Kaplan and Lipmann (9). Total nitrogen was determined by the procedure of Hiller, Plazin, and Van Slyke (10) on aqueous homogenates of tissue prepared for coenzyme A assay. Dry weights were determined on the same homogenates by drying an aliquot overnight at 115°.

Results

Data on each group of rats, showing the average food intake, initial body weight, and change in body weight over the 10 day period of observation, are presented in Table I. Liver coenzyme A is expressed in terms of Kaplan-Lipmann units of coenzyme A per gm. of wet weight, per 100 gm. of body weight, per 100 mg. of total homogenate nitrogen, and per 100 mg. of dry weight.

As has been previously observed, hypophysectomy greatly reduced the food intake of the immature rat, and such animals failed to gain weight over the 10 day experimental period. Hypophysectomized rats stimulated with growth hormone gained 15 gm. in body weight and increased their food consumption approximately 10 per cent. Normal adult rats gained 5 gm., while normal adult rats stimulated with growth hormone gained 14 gm.

The concentration of liver coenzyme A expressed on the basis of 100 gm. of body weight was found to be significantly lower in both hypophysectomized immature and normal adult rats than in rapidly growing

immature normal rats.[2] Stimulation of the normal adult rat with growth hormone resulted in a significant elevation in liver coenzyme A in this animal, while stimulation of the hypophysectomized immature rat with growth hormone resulted in a somewhat further but insignificant reduction in the level of liver coenzyme A in this animal.

TABLE I

Liver Coenzyme A in Normal and Induced States of Growth[*]

Group	No. of rats in group	Body weight at start of 10 day experimental period	Change in body weight over 10 day period	Daily food intake	Terminal liver weight	Liver coenzyme A, Kaplan-Lipmann units			
						Per gm. wet weight	Per 100 gm. body weight	Per 100 mg. total homogenate N	Per 100 mg. dry weight
		gm.	*gm.*	*gm. per day*	*gm.*				
Normal, immature	6	169	+19	13.1	7.18	150 (±26)	559 (±91)	465 (±81)	50 (±11.4)
Hypophysectomized, immature	9	103	+0	5.2	3.27	127 (±22)	406 (±70)	364 (±69)	45 (±11.1)
Hypophysectomized, immature, stimulated with growth hormone†	11	111	+15	5.8	4.11	119 (±15)	387 (±42)	340 (±53)	40 (±11.7)
Normal, adult	6	281	+5	12.8	7.68	106 (±4.4)	284 (±21)	324 (±23)	34 (±6.1)
" " stimulated with growth hormone†	6	310	+14	13.7	9.23	122 (±16)	347 (±41)	373 (±44)	42 (±5.0)

* The values tabulated are averages calculated for each group of animals. The values in parentheses are standard deviations.

† Preparation and administration of growth hormone described under "Methods."

Results of coenzyme A determinations on kidney tissue obtained from the same series of rats are summarized in Table II. The concentration expressed on the basis of gm. of wet weight, per 100 mg. of total homogenate nitrogen, and per 100 mg. of dry weight was not significantly al-

[2] Data expressed on the basis of units of tissue coenzyme A per 100 gm. of body weight were selected for statistical analysis, since it was felt that a better comparison of total coenzyme A concentration in animals of different weights would be obtained. The methods of Fisher (11) were used in analyzing the data, and P values <0.01 were considered significant.

tered by hypophysectomy of the immature rat, by cessation of growth in the normal rat as a result of attainment of the adult state, or by stimulation of the growth process in either the hypophysectomized immature or normal adult rat with growth hormone. Although coenzyme A concentration expressed on the basis of 100 gm. of body weight was found to be significantly higher in hypophysectomized immature rats and significantly lower in normal adult rats than in normal immature animals, there appears to be no relationship to the growth process. Stimulation of

TABLE II

*Kidney Coenzyme A in Normal and Induced States of Growth**

Group†	No. of rats in group	Terminal kidney weight	Kidney coenzyme A, Kaplan-Lipmann units			
			Per gm. wet weight	Per 100 gm. body weight	Per 100 mg. total homogenate N	Per 100 mg. dry weight
		gm.				
Normal, immature	6	0.689	49 (±9.8)	18.3 (±3.2)	174 (±28)	23.2 (±6.8)
Hypophysectomized, immature	9	0.557	47 (±7.6)	25.2 (±6.2)	161 (±38)	21.5 (±4.4)
Hypophysectomized, immature, stimulated with growth hormone	11	0.632	51 (±5.9)	26.2 (±5.1)	168 (±29)	23.1 (±3.4)
Normal, adult	6	0.836	45 (±4.7)	13.1 (±1.8)	166 (±24)	20.6 (±2.3)
" " stimulated with growth hormone	6	0.942	47 (±10.0)	14.0 (±3.5)	176 (±44)	23.9 (±0.6)

* The values tabulated are averages calculated for each group of animals. The values in parentheses are standard deviations.

† Same series of animals described in Table I.

either hypophysectomized immature or normal adult rats with growth hormone produced no significant alteration in the level of kidney coenzyme A in these animals.

DISCUSSION

Results of studies on the pantothenic acid requirement of the rat (12) seem to indicate that the normal adult requires less dietary pantothenic acid than the rapidly growing immature rat. This general observation and the finding that metabolically active pantothenic acid is largely bound in coenzyme A (4) suggest that the metabolic requirement for coenzyme A might also be similarly related to the growth process. The results of our studies on the concentration of coenzyme A in liver tissue

obtained from rapidly growing immature normal rats, from normal adult rats, and from normal adult rats stimulated with growth hormone, as well as the results of studies on the concentration of liver coenzyme A in hypophysectomized immature rats, appear to be in complete agreement with this suggestion. Stimulation of the hypophysectomized rat with growth hormone, however, failed to alter the concentration of coenzyme A significantly in liver tissue obtained from this animal, even though the rat was known to be storing nitrogen and gaining body weight at a rate which approached that for rapidly growing immature normal animals. A priori, this anomaly in the otherwise direct relationship between liver coenzyme A and the growth process might be considered to be due to the lack of tropic hormones other than growth hormone. In this respect the work of Tabachnick and Bonnycastle (13) on the effects of thyroidectomy and thyroxine on the concentration of coenzyme A in liver tissue is particularly suggestive. Thyroidectomy resulted in a significant reduction in the level of liver coenzyme A, and stimulation of the thyroidectomized rat with thyroxine restored concentrations of liver coenzyme A to normal or above. Since the hypophysectomized rat is essentially hypothyroid due to the lack of thyrotropic hormone, the inability of the hypophysectomized immature rat stimulated with growth hormone to increase the concentration of coenzyme A in its liver tissue might well be due to thyroxine deficiency.

In view of the greatly reduced food intake of hypophysectomized immature rats stimulated with growth hormone in comparison with that of rapidly growing immature normal rats, it might also be suggested that a suboptimal dietary intake or perhaps an alternative metabolic channeling of pantothenic acid and amino acids required for coenzyme A biosynthesis could be considered explanatory of the negative effect of growth hormone on liver coenzyme A in the hypophysectomized rat.

Results of studies concerning the effects of pantothenate and amino acid supplementation on liver coenzyme A in hypophysectomized rats stimulated with growth hormone, as well as studies on similar rats supplemented with thyroxine, will form the basis of subsequent reports.

SUMMARY

Concentrations of liver coenzyme A were found to be significantly lower in both hypophysectomized immature and normal adult rats than in rapidly growing immature normal rats. Induction of nitrogen storage and gain in body weight in the normal adult rat as a result of stimulation with anterior pituitary growth hormone are accompanied by a significant elevation in the concentration of coenzyme A in the liver of this animal. Stimulation of the growth process in the hypophysectomized rat with

growth hormone does not significantly alter the level of liver coenzyme A in this animal. Concentrations of kidney coenzyme A do not appear to be significantly altered by hypophysectomy of the immature rat, by cessation of growth in the normal rat as a result of attainment of the adult stage, or by stimulation of the growth process in either the hypophysectomized immature rat or the normal adult rat with growth hormone. There appears to be a direct relationship between liver coenzyme A and growth in the normal rat.

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CARBOHYDRATE OXIDASE FROM A RED ALGA, IRIDOPHYCUS FLACCIDUM*

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Enzymes capable of oxidizing D-glucose to D-gluconic acid are known to be present in bacteria (1, 2), fungi (3), and animal tissues (4, 5). However, no such enzyme has hitherto been reported in a photosynthetic organism. It was observed in the course of a study of the carbohydrate metabolism of the marine red alga, *Iridophycus flaccidum* (6), that cell-free extracts of this plant had the ability to oxidize D-glucose to D-gluconic acid. Further investigation revealed that this preparation will also oxidize D-galactose to D-galactonic acid, and several reducing disaccharides, namely maltose, lactose, and cellobiose, to their corresponding aldobionic acids. It therefore appears that this oxidase differs from other D-glucose oxidases in that it can utilize a variety of sugar substrates.

The present communication is concerned with the preparation of active oxidase fractions from this red alga, the purification, and study of the properties of the enzyme.

EXPERIMENTAL

Assay Methods—A standard Warburg manometric method (7) was employed for assay of the enzyme. An aliquot of 0.01 to 1.0 ml. of the enzyme solution, together with 1.0 ml. of a 0.5 M maleate buffer, pH 5.2, was placed in the main chamber of the Warburg vessel and diluted with water to a volume of 2 ml. A 0.5 ml. aliquot of 0.5 M solution of D-glucose (saturation of the enzyme occurs at approximately 0.02 M glucose) or other carbohydrate used as substrate was placed in the side arm. Since no carbon dioxide was evolved in the course of the reaction, it was not necessary to use alkali in the center well of the main chamber. Materials which were tested as inhibitors or activators were mixed with the enzyme in the main chamber or added from a second side arm, depending on the conditions of the experiment. Hydrogen peroxide was generated during the reaction. In the absence of added catalase the peroxide was not

decomposed. Determinations were made without the addition of catalase in order to take advantage of the greater rate of oxygen uptake in the absence of this enzyme. An enzyme unit of oxidase was the amount of enzyme required to cause the uptake of 1 μmole of oxygen per hour at 30°. This value of oxygen uptake also corresponded to the oxidation of 1 μmole of D-glucose to D-gluconic acid per hour.

In addition to the manometric assay, a photometric determination for the enzyme, with 2,6-dichlorophenol-indophenol, was occasionally employed. This assay proved to be useful to rapid checks of oxidase activity during the process of fractionation. However, since the photometric assay could not be carried out at pH 5, which is the optimum of the reaction, it was not generally used.

Analysis for the gluconolactone was made by the method of Hestrin (8). The enzymatic reaction was carried out for varying periods in Warburg flasks in arsenate buffer (the maleate buffer interfered with the lactone determination) at pH 5. The reaction was then stopped by the addition of the hydroxylamine in alkaline solution and the hydroxamic acid formation carried out according to Hestrin's procedure.

Extraction Procedure and Fractionation of Enzyme Preparations—Fresh *I. flaccidum* (6) plants were rinsed with distilled water, the excess water was wiped off, and the material placed in a cold room at −12° for 2 to 4 hours. The partially frozen thalli were ground in the cold with a hand meat grinder having 0.25 inch apertures, 1 kilo of the coarsely ground material was taken up in 1500 ml. of distilled water, and the macerated tissue allowed to stand overnight at 0–4°. After infiltration of the ground material by squeezing through four layers of cheese-cloth, 1050 ml. of solution were obtained. Upon extraction of the solid residue with 1 liter of water and filtration through a cheese-cloth as before, another liter of solution was obtained. The extracts, having almost identical oxidase activity, were combined, and the extracted residue, which still contained a small amount of activity, was discarded. Before the barium-methanol fractionation, the extract was dialyzed overnight against tap water.

Barium-Methanol Fractionation—60 gm. of $BaCl_2 \cdot 2H_2O$ were dissolved in 350 ml. of the dialyzed red alga extract and the solution was frozen overnight. The frozen extract was allowed to thaw, the insoluble material was removed by centrifugation (10,000 × g for 10 minutes), the solution cooled in ice to 1°, and 35 ml. of methanol at −10° were added over a period of 15 minutes. The precipitate of low activity was removed by centrifuging for 10 minutes at 0°. The supernatant solution was treated with another 115 ml. of cold methanol, the temperature being kept below 2°. After standing for half an hour with occasional stirring, the second precipitate was removed by centrifugation.

The third supernatant solution was further treated with a slow addition of 200 ml. of methanol so that the temperature did not rise above 2°. After centrifuging for 10 minutes in the cold, the precipitate (Fraction 1, Table I) was suspended in 10 ml. of water, dialyzed against tap water for 2 hours, and then overnight at 1° in distilled water. This fraction (11 ml.) had an activity of 332 units per ml.

After precipitation of Fraction 1, the supernatant solution was devoid of enzymatic activity. The first two methanol precipitates could be resolved into an inactive component and a fraction with a specific activity approximating that of Fraction 1 by subjecting them to a repetition of the above procedure.

TABLE I

Enzymatic Activities of Extracts and Fractions

For the conditions of substrate oxidation, see the text, "Assay methods."

Enzyme preparation	Enzyme units* per ml.	Total volume, ml.	Total enzyme units	Enzyme units* per mg. N	Enzyme units* per mg. dry weight†
Plant extract.....................	20	350	7000	80	3.9
Fraction 1, barium-methanol ppt.	332	11	3870	580	40
" 2, (NH₄)₂SO₄ ppt........	437	7	3060	1060	141

* The enzyme unit is the amount of enzyme required to cause the uptake of 1 μmole of oxygen per hour.

† The specific activities of the preparations are expressed on a dry weight basis as well as on the basis of mg. of nitrogen in order to show the extent of the removal of the contaminating polysaccharide at different stages of purification.

Fraction 1 was very stable, one sample retaining 90 per cent of its activity when stored for 7 months at −10°.

Ammonium Sulfate Fractionation—Further purification of Fraction 1 was effected by an ammonium sulfate fractionation. The barium-methanol fraction (11 ml.) was cooled to 0°, 8 ml. of cold saturated ammonium sulfate were added slowly with stirring, and, after allowing the mixture to stand for an hour, the precipitate was removed by centrifugation. The activity of this fraction was very low.

The supernatant solution was further treated with 6 ml. of saturated ammonium sulfate in the cold. The precipitate (Fraction 2) was collected, dissolved in water, and dialyzed overnight against cold, distilled water. Fraction 2 had a final volume of 7 ml. and an activity of 437 units per ml. and of 1060 units per mg. of N, which amounted to a 13-fold increase in activity over that of the dialyzed extract. The nitrogen content of the dried material was 13.3 per cent, indicating that most of the con-

taminating polysaccharide had been removed at this stage of purification. Expressed on dry weight basis, the final "purification" amounted to a 36-fold increase in activity. Fraction 2 was not as stable as the less purified fractions. Most of its activity was lost on further attempts of purification. On remaining frozen at −20° for a month, it lost 50 per cent of its activity.

Oxidation of Sugars and Identification of Aldonic Acids—The D-gluconic and D-galactonic acids derived from the oxidation of D-glucose and D-galactose, respectively, by the oxidase were identified by a combination of radioactive tracer and paper chromatographic techniques. In addition, the oxidation product of D-glucose was isolated as the barium gluconate salt and as crystalline D-gluconolactone, and the compounds were identified by their physical and chemical properties.

C^{14}-labeled glucose, galactose, and maltose were incubated with the enzyme and the products were identified chromatographically by comparing with synthetic samples of gluconic, galactonic, and maltobionic acids, procedures previously described being used (6). The results showed that the corresponding aldonic acid was formed from each sugar in all three cases. The chromatographic patterns of the reaction products were found to be identical with those of D-gluconic, D-galactonic, and maltobionic acids, which had been prepared by bromine oxidation of the radioactive D-glucose, D-galactose, and D-maltose, respectively (9). Hydrolysis of the enzymatically formed maltobionic acid produced glucose and gluconic acids.

The formation of gluconic acid in the oxidation of the C^{14}-labeled glucose was further confirmed by degradation of the chromatographically isolated oxidation product to arabinose by treatment with hydrogen peroxide in the presence of a ferric acetate catalyst (10).

In another experiment 250 mg. of glucose were incubated with the enzyme. Barium gluconate was isolated from the reaction mixture and the product recrystallized from methanol and water to give 95.1 mg. of the pure salt. This enzymatically prepared crystalline form had a melting point of 147–148°, and a specific rotation, $[\alpha]_D$ +7.9° (c, 1 in water). These constants are in good agreement with those found for authentic gluconate which had been crystallized in the same manner.

Gluconolactone was prepared from pooled barium gluconate fractions by a modification of the method of Isbell *et al.* (11). A yield of 39.8 mg. of the lactone was obtained. Polarization of this lactone showed an initial $[\alpha]_D$ +65.9° after 8 minutes, which gradually changed to a constant value of 7.6° after 6 hours. These values agreed closely with those of D-gluconolactone obtained by other workers (12).

The primary product of the reaction was actually the lactone of the

aldonic acid rather than the free acid identified above. That this was the case was shown in two ways. When the enzyme was allowed to act on glucose in the absence of buffer in an oxygen atmosphere, there was a slow change in pH at the beginning of the reaction. After replacing the oxygen with nitrogen to stop the reaction, the gradual drop in pH continued to occur, indicating that an acidic group was still being liberated even in the absence of oxidation. It was also possible to show formation of hydroxamic acid from the lactone, when the oxidation was carried out for 15 minutes and then alkaline hydroxylamine was added to stop the
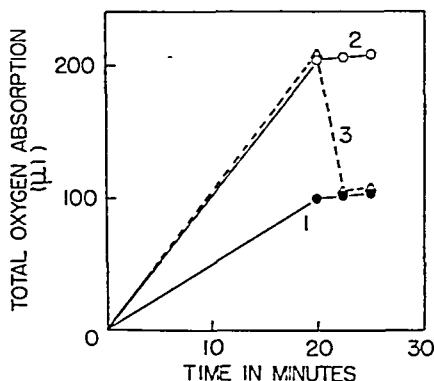
FIG. 1. Stoichiometry of glucose oxidation and H_2O_2 production. All manometer vessels contained 1 ml. of 0.5 M maleate buffer, pH 5.2, and 1 ml. of enzyme solution in the main chambers, and 0.5 ml. of 0.5 M D-glucose solution in one of the side arms. Vessel 1 (Curve 1) also contained catalase in the main chamber. After mixing of the buffered enzyme solution with the D-glucose in the manometer vessels and allowing the reaction to proceed for 20 minutes, 0.5 ml. of 1 M sodium acetate solution (oxidase inhibitor), pH 5, was added to Vessels 1 and 2 (Curves 1 and 2), and sodium acetate plus catalase to Vessel 3. The reaction is arrested by the acetate, while the catalase destroys the hydrogen peroxide formed in Vessel 3.

reaction. The analysis for hydroxamic acid formation was carried out by the method of Hestrin (8). When the oxygen uptake indicated formation of 11.6 and 16.4 μmoles of gluconate, 8.7 and 13 μmoles of hydroxamic acid were found to be present, respectively. The fact that the enzyme will oxidize disaccharides having the C-4 hydroxyl engaged in the glycosidic linkage (see "Substrate specificity") indicates that the δ-lactone rather than the γ-lactone is formed in the reaction.

Properties of Oxidase—When the oxidase was allowed to act on D-glucose in the Warburg apparatus, hydrogen peroxide was found to be produced during the reaction. The stoichiometry between oxygen uptake and hydrogen peroxide formation is demonstrated in Fig. 1. The rate of oxygen absorption in the samples in Vessels 2 and 3 (Curves 2 and 3) is

twice as great as that in Vessel 1 to which catalase had been added at the beginning of the experiment. When catalase was added after 20 minutes to Vessel 3 in the presence of an oxidase inhibitor (sodium acetate), oxygen was rapidly evolved equivalent to half that consumed in the absence of catalase. This indicates that the samples are completely free of catalase and that 1 mole of hydrogen peroxide is formed per mole of oxygen consumed.

The enzyme can utilize 2,6-dichlorophenol-indophenol as a hydrogen acceptor; in the presence of oxygen, both the dye and oxygen are reduced. Oxygen appears to be reduced more readily than the dye. Other compounds, such as methylene blue, tetrazolium violet, or ferricyanide in ranges from 10^{-5} to 10^{-3} M which usually react with flavoproteins, are not reduced by this enzyme under anaerobic conditions. The oxidase does not require the addition of any coenzymes and retains its full activity after long periods of dialysis. Attempts to split the protein from the prosthetic group by treatment with acid and then to reconstitute the enzyme were not successful. When the enzyme was treated with acid in the presence of ammonium sulfate at pH 3, there was no loss of activity in the precipitated protein. At pH 2 the enzymatic activity was completely lost and could not be restored by the addition of flavin adenine dinucleotide or with concentrates of a supernatant solution from boiled enzyme preparations. Dialysis against dilute acid (0.01 M) also destroyed the activity irreversibly.

Absorption spectra given by the partially purified enzyme and by the split-products after acid treatment of the enzyme did not show the characteristic flavin absorption maxima peaks at 450 and 380 mμ. Strong absorption maxima at 265 and 330 mμ were given by the split-products. However, there was no correlation between the material responsible for these absorption peaks and the enzymatic activity in the fractionations; the same absorption peaks were found to be even more pronounced in some inactive fractions.

Substrate Specificity—The following carbohydrates were tested as possible substrates: D-glucose, D-galactose, maltose, lactose, cellobiose, L-glucose, D-fructose, D-mannose, melibiose, raffinose, L-fucose, L-arabinose, D-xylose, D-galacturonic acid, D-glucuronolactone, D-glucose-6-phosphate, dulcitol, glycerol, glycerol phosphate, α-methyl-D-glucoside, and 2,3-methyl-D-glucose. Of these compounds only the first five listed were found to be attacked by the oxidase preparations (see Table II).

All the organic compounds tested as inhibitors (Table IV) were also examined to determine whether they caused oxygen absorption or carbon dioxide evolution in the presence of the enzyme under normal conditions of enzymatic oxidation. None of these substances were found to be active in this respect.

Table II represents the rate of oxidation of five carbohydrates found to be attacked by the oxidase preparation. That the same enzyme is probably responsible for the oxidation of both D-glucose and D-galactose (and possibly for the three disaccharides) is shown by the fact that the ratio of the rates of oxidation of D-glucose and D-galactose is constant at all stages of the enzyme purification (Table III). An experiment in which saturating amounts of both D-glucose and D-galactose were added to the incuba-

TABLE II

Rates of Oxidation of Various Carbohydrate Substrates by I. flaccidum Oxidase

For conditions of substrate oxidation, see the text, "Assay methods." 0.1 M concentrations of sugars were used.

Substrate	Micromoles O_2 per min. per ml. enzyme
D-Glucose	4.3
D-Galactose . .	4.3
Lactose	2.1
Maltose... .	2.0
Cellobiose ...	1.3

TABLE III

Comparison of Rates of Oxidation of D-Glucose and D-Galactose at Different Stages of Enzyme Fractionation

0.1 M concentrations of D-glucose and D-galactose were used, and the oxidations were carried out as described under "Assay methods." The values are given in micromoles of O_2 per hour per ml. of enzyme.

Enzyme preparation	D-Glucose	D-Galactose	Activity ratio, D-galactose to D-glucose
Extract	20	18	0.90
Methanol ppt.	40	34	0.85
Fraction 1, barium-methanol	332	300	0.90
Fraction 2, ammonium sulfate	437	390	0.89

tion mixture showed that no increase in the rate of oxygen uptake occurred over that found when D-glucose alone was present.

The Michaelis constant for D-glucose was found to be 0.0025 M and that for D-galactose 0.005 M. These constants are of the same order of magnitude as K_m for D-glucose with notatin, which is given by Keilin and Hartree (3) as 0.0042 M.

The disaccharides appear to be oxidized directly without preliminary hydrolysis. This is shown by the formation of maltobionic acid from maltose and the fact that, when the reactions are allowed to proceed to completion, the total oxygen consumption with the disaccharides is equal to that obtained with equimolar amounts of the monosaccharides.
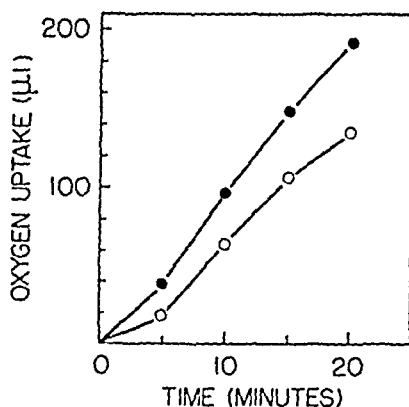
FIG. 2. Comparison of enzymatic action on α and β isomers of glucose. O, α-D-glucose; ●, β-D-glucose. 0.75 ml. of arsenate buffer, 0.25 ml. of enzyme solution, and 2 mg. of dry, crystalline α- or β-D-glucose were added from the side arm at zero time to give 0.011 M concentration. The β-D-glucose contained 85 per cent of the β isomer.

TABLE IV

Inhibition of Sugar Dehydrogenase from I. flaccidum

Compound	Per cent inhibition*,†			
	10^{-5} M	10^{-3} M	10^{-2} M	10^{-1} M
Mercuric chloride........ ..	5‡	100		
Lead acetate........		100		
Silver nitrate............ . .	50	100		
p-Chloromercuribenzoate.... .	2–5	25§		
Acetate, sodium..		21	63	95
Propionate, sodium.....		19	60	90
Pyruvate, potassium......			20	
Benzoate, sodium..............			100	
D-Mannose†................				40

Assay conditions, except in the case of D-mannose and D-xylose: 1 ml. of maleate buffer, 0.5 ml. of enzyme solution in the main chamber of the manometer vessel; 0.5 ml. of 0.5 M D-glucose in Side Arm 1; 0.5 ml. of inhibitor test solution in Side Arm 2. Rates of oxygen uptake were checked with D-glucose alone in each experiment, and then the inhibitor was added from Side Arm 2 to determine the inhibited rate.

* Other compounds were tested which caused no inhibitory effects: 10^{-2} M concentrations of copper sulfate, aluminum sulfate, zinc chloride, magnesium chloride, calcium chloride, sodium formate, sodium butyrate, sodium lactate, sodium glycolate, sodium glyoxylate, sodium citrate, sodium succinate, sodium Versenate, hydroxylamine hydrochloride; 10^{-1} M barium chloride, and D-xylose and acetaldehyde.

† The glucose in the mannose and xylose experiments was added as 0.01 M solution to give a final concentration of 0.002 M instead of the 0.1 M final concentration used in the rest of the experiments.

‡ Mercuric chloride at 10^{-4} M gave 60 per cent inhibition.

§ Actual concentration of p-chloromercuribenzoate was 2×10^{-4} M.

When the α and β isomers of D-glucose were tested with the enzyme at low concentrations, it was found that the β-D-glucose was oxidized at a slightly higher rate than the α isomer (Fig. 2).

Inhibitor Action—The compounds tested for inhibition of the enzymatic activity are listed in Table IV. Although Hg^{++}, Ag^+, Pb^{++}, and p-chloromercuribenzoate do not inhibit the enzyme, it is doubtful that the slight inhibitory effects at low concentrations are connected with any specific
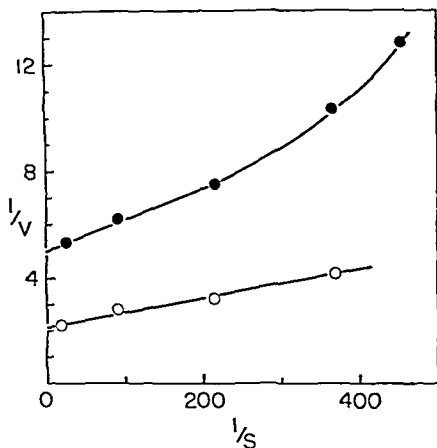
FIG. 3.
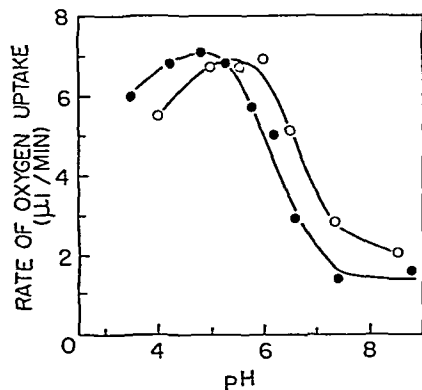
FIG. 4.

FIG. 3. Inhibition of dehydrogenase action by acetate. O, glucose; ●, glucose + acetate. The main chamber of the manometer vessel contained 0.5 ml. of 0.5 M maleate buffer, pH 5.1, 0.2 ml. of enzyme solution, 0.3 ml. of water in the controls, or 0.3 ml. of 0.05 M sodium acetate, pH 5, in the inhibited series. The side tubes contained 0.5 ml. of D-glucose solutions of dilutions to give the desired final concentrations. Lineweaver-Burk plot (13) of data. S, the substrate concentration in moles per liter; V, the initial rate in micromoles of O_2 per minute.

FIG. 4. Change of oxidase activity with pH; ●, phosphate buffer, 0.5 M; O, phosphate-citrate buffer, 0.5 M. The conditions for pH-activity determination are described in the text under "Optimal pH."

effect involving an active enzyme group. No other metals tested had any inhibitory effect on the enzyme. The lack of inhibition by Versene indicates that a metal is probably not connected with the activity unless it has formed a very firm complex with the protein.

The inhibition of the enzyme by the anions, acetate, propionate, benzoate, and pyruvate cannot be explained at this time. Since formic and butyric acids have no effect on the activity of the enzyme, it appears that the inhibition is specifically limited to the 2- and 3-carbon members of the fatty acid group. When acetate or propionate was used, it made no difference in the degree of inhibition whether the source of the anion was the crystalline salt or the free acid. Thus it is probable that the acetate and

propionate themselves, rather than some impurity, are responsible for the inhibition. As shown in Fig. 3, the effect of acetate is non-competitive with D-glucose.

D-Mannose, but not D-xylose, acts as a weak competitive inhibitor of the reaction with D-glucose. The effect of D-mannose is perceptible only at fairly high ratios of D-mannose to D-glucose.

Optimal pH—The pH optimum for the oxidase was determined by using phosphate-citrate buffer as well as phosphate buffer alone, adjusted to the desired pH. 1 ml. of the enzyme solution was added to 1 ml. of the
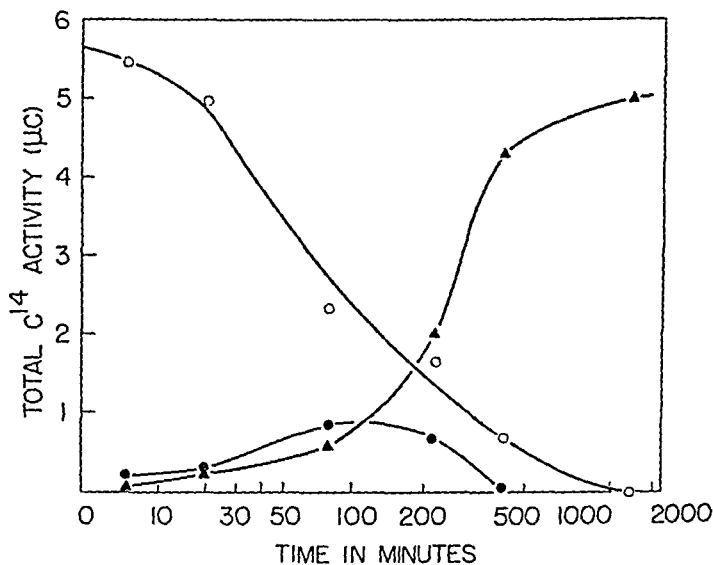
FIG. 5. Oxidation of glucose *in vivo* by *I. flaccidum*. O, glucose; ●, unidentified compound; ▲, gluconic acid. For each time point in the chart, 0.14 mg. of C^{14}-labeled glucose (42 μc. per mg.) in 0.2 ml. of water was incubated with two disks of 90 mg. each of the thallus for a given time period. The disks were inactivated and extracted with hot alcohol. Aliquots of the extracts were analyzed by paper chromatography in two dimensions and the radioactivity in each component was counted directly on paper.

buffer, and the pH was then checked on a Beckman pH meter. Since in some cases the pH changed with the progress of the reaction due to formation of gluconic acid, in order to obtain the initial rate of reaction it was necessary to extrapolate the oxygen uptake curve for each pH value to zero time to determine the true value for the rate at that pH. The data show that the pH optimum for the reaction is about 5.0 (Fig. 4). Slow denaturation of the enzyme occurred at pH values above 7.5.

Investigation of Oxidase in Vivo—Experiments similar to those performed by Putman and Hassid (14), in which C^{14}-labeled glucose was infiltrated into actively metabolizing disks, demonstrated that the oxidase functioned *in vivo*. The results on infiltrating C^{14}-labeled D-glucose are illustrated in Fig. 5. The data show a gradual diminution of activity in glucose and a

simultaneous appearance of activity in gluconic acid and in a transient, unidentified compound. At the end of the reaction most of the activity was found in gluconic acid.

The results with infiltrated C^{14}-labeled D-galactose were similar to those obtained with radioactive D-glucose. The aldonic acid and an unidentified compound were simultaneously produced. At the end of the incubation period the activity was found to reside almost quantitatively in the galactonic acid and its lactones.

Gluconic acid was also identified as one of the C^{14}-labeled products when the plant was allowed to photosynthesize in the presence of $C^{14}O_2$ (6).

DISCUSSION

Two systems which oxidize D-glucose to D-gluconic acid have been thoroughly studied. The first, D-glucose oxidase (notatin), which exists in *Penicillium notatum* and possesses flavin adenine dinucleotide as its prosthetic group (3), is quite specific for D-glucose; no other sugar is oxidized by this enzyme at a rate greater than 1 per cent of that of D-glucose. Sodium nitrite, β-hydroxyquinoline, and semicarbazide are the only substances found to have an appreciable inhibitory effect on notatin. The second, the mammalian D-glucose dehydrogenase system (4), depends upon the pyridine nucleotides as its coenzymes. This enzyme is also highly specific for D-glucose, although it attacks D-xylose to some extent (5). It is inhibited by a low concentration of heavy metals. A third system, which depends upon a modified cytochrome transport path (1), is found in *Pseudomonas fluorescens*. This system is specific for D-glucose and is strongly inhibited by cyanide.

Since the generation of hydrogen peroxide is frequently associated with flavoprotein oxidation, it is probable that this enzyme contains a flavin coenzyme. However, spectrographic analysis of the enzyme preparation did not show the presence of a flavin group. The inability to demonstrate this group may be attributed to the low purity of the enzyme preparation. Comparing the rate of oxidation of D-glucose by this impure oxidase preparation with that by pure notatin on dry weight basis, it is found that the former is about 25 times less active than the latter. In its behavior toward heavy metals, the *Iridophycus* enzyme appears to be similar to the mammalian dehydrogenase. However, in its substrate specificity it differs radically from all the other known oxidases. The fact that the *Iridophycus* oxidase is capable of oxidizing D-glucose and D-galactose but not D-mannose indicates that the enzyme is specific with regard to the C-2 configuration and is non-specific with regard to that of C-4 in the hexose chain. Since this enzyme is also capable of oxidizing the disaccharides, lactose, maltose, and cellobiose, it can be concluded that the

hydroxyl of C-4 may be substituted by a D-glucose or D-galactose unit without greatly affecting the activity of the enzyme. A 6-carbon sugar is apparently required, since the enzyme shows no activity toward D-xylose or L-arabinose, which are the pentose analogues of D-glucose and D-galactose, respectively. Inasmuch as modification of the hydroxyl group at C-6 of D-glucose by substitution, as in melibiose, or by oxidation of C-6 of D-galactose, as in galacturonic acid, prevents the enzymatic reaction from taking place, the primary C-6 hydroxyl appears to be indispensable.

The function of direct oxidation of sugars in living cells is not clear. Although there is evidence that the gluconate produced in the red alga is slowly utilized, its rate of accumulation appears to exceed greatly that of utilization.

SUMMARY

A cell-free enzyme preparation capable of oxidizing D-glucose to D-gluconic acid, D-galactose to D-galactonic acid, and the disaccharides, maltose, lactose, and cellobiose, to their corresponding aldobionic acids has been obtained from the red alga *Iridophycus flaccidum*. In these reactions oxygen is consumed and H_2O_2 is formed. The optimal pH for the oxidation is approximately 5.0.

The enzyme appears to be specific with regard to the C-2 configuration of the hexose chain. It remains active when the C-4 configuration is changed or when the hydroxyl on this carbon is substituted by a D-glucose unit.

The enzyme from the algal extract was partially purified by fractionation with methanol in the presence of barium followed by ammonium sulfate precipitation.

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A MAMMALIAN 3α-HYDROXYSTEROID DEHYDROGENASE

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The reduction of α,β-unsaturated 3-ketosteroids to their corresponding saturated 3-alcohols comprises a major pathway of metabolism of C_{19} and C_{21} steroids (1). A preliminary report from this laboratory (2) described a cell-free preparation of rat liver capable of catalyzing the two-step reduction of cortisone to tetrahydrocortisone with reduced pyridine nucleotides as hydrogen donors. The first reaction involves the hydrogenation of the double bond of ring A by TPNH[1] to produce dihydrocortisone. The present communication deals in detail with the second step of the proposed mechanism, *i.e.* the interconversion of dihydrocortisone and tetrahydrocortisone as follows:

$$DHC + DPNH + H^+ \rightleftharpoons THC + DPN^+$$
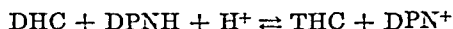
It is shown that the enzyme catalyzing this reaction is also involved in the formation of other 3α-hydroxysteroids, and therefore is similar to the bacterial 3α-hydroxysteroid dehydrogenase studied by Talalay and Marcus (3).

Materials and Methods

Substrates—Dihydrocortisone, pregnane-3α,11β,17α,21-tetrol-20-one, and cortisone were generously donated by Merck and Company. Etiocholane-3,17-dione and androstan-3β-ol-17-one were kindly supplied by Dr. Ralph Peterson. Androstane-3,17-dione, androstan-17β-ol-3-one, cholestan-3-one, coprostan-3-one, and Δ^1-androstene-3,17-dione were obtained from the Chemed Manufacturing Company, White Plains, New York. All steroids employed were chromatographically pure.

Preparation of Substrate Solutions—Because of their relative insolubility in water, supersaturated solutions of the steroids were made as follows: Either a known quantity of the compound was dissolved in a small volume of absolute methanol which was diluted rapidly with sufficient hot water

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[1] The abbreviations used in this communication are DHC (dihydrocortisone), THC (tetrahydrocortisone), DPN$^+$ and DPNH (oxidized and reduced diphosphopyridine nucleotides, respectively), TPN$^+$ and TPNH (oxidized and reduced triphosphopyridine nucleotides, respectively), Tris (tris(hydroxymethyl)aminomethane), PCMB (*p*-chloromercuribenzoate), and PO$_4$ (sodium phosphate).

to make a final methanol concentration of 10 per cent (volume per volume), or boiling water was added directly to the dry steroid and the mixture was shaken vigorously in a mechanical shaker for 5 minutes, following which any material not dissolved was removed by centrifugation. In each case, the concentration of the dissolved steroid was determined by an appropriate procedure. For 17-hydroxycorticosteroids, a modified Porter-Silber reagent was used (4), and for 17-ketosteroids the Zimmermann reaction was employed (5).

Coenzymes—DPN⁺ "90," TPN⁺ "80," DPNH "90," and TPNH "90" were obtained from the Sigma Chemical Company, St. Louis, Missouri. TPNH was also prepared by the hydrosulfite reduction procedure of Kaplan *et al.* (6). The extinction coefficient of reduced pyridine nucleotide at 340 mμ was taken to be 6.22×10^6 sq. cm. per mole (7). The concentration of DPN⁺ and TPN⁺ was determined spectrophotometrically by reduction with glucose in the presence of the purified beef liver glucose dehydrogenase of Strecker and Korkes (8). The DPNH concentration was determined spectrophotometrically by its oxidation with pyruvate and lactic dehydrogenase.[2]

Enzyme Assay—The activity of the dehydrogenase was calculated from the initial rate of DPNH oxidation measured at 340 mμ in a Beckman model DU spectrophotometer. The reaction was carried out in quartz cells having a 1.0 cm. light path and a 1.3 ml. capacity. Normally the reaction velocity was linear with time for 45 seconds and an enzyme preparation was defined as that having 1 unit of activity if it caused a decrease in optical density of 0.01 per minute measured during the interval 15 to 30 seconds after addition of the enzyme to the reaction mixture. Specific activity was defined as units of activity per mg. of protein. Protein was estimated according to Warburg and Christian (9).

Fig. 1 shows the dependence of reaction velocity on enzyme concentration. Only the linear portion of the curve (less than 1 mg. of protein) was used for enzyme assay. The deviation from linearity at higher protein concentrations was assumed to be due to the fact that at increased reaction velocities the 15 to 30 second interval used in the assay did not represent the true initial rate but gave a falsely low value. In the purified enzyme preparation, there was no DPNH oxidation or DPN⁺ reduction in the absence of substrate. In cruder preparations these interfering reactions were either absent or present to such a slight degree that they represented at most 5 per cent of the enzyme activity being measured.

Results

Purification of Enzyme—The dehydrogenase was present in rat liver, kidney, and testis, but not in the other rat organs tested (Table I). A

2 Kindly supplied by Dr. W. W. Kielley.

fresh dog liver preparation was active initially but deteriorated completely within 2 hours at 0°. Rabbit liver extracts were also active, but no significant activity was detected in extracts of beef, hog, guinea pig, mouse, or human liver, perhaps because of pronounced lability such as was observed in the dog. Rat liver was selected as a convenient source of the enzyme.
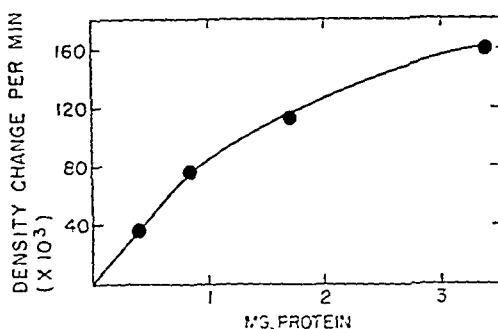
FIG. 1. Enzyme-velocity curve. The cuvettes contained 0.05 μmole of DPNH, 0.11 μmole of DHC, 20 μmoles of PO_4 buffer, pH 7.4, and enzyme in a total volume of 1.1 ml. The reaction was started by the addition of the enzyme.

TABLE I

Distribution of 3α-Hydroxysteroid Dehydrogenase in Rat Tissue

Tissue	Specific activity* of tissue extract
Liver	26.8
Kidney	9.8
Testis	6.2
Lung	0
Spleen	0
Muscle	0
Brain	0

* Units per mg. of protein.

Rats were killed by a sharp blow on the head and their livers were rapidly excised and chilled. They were then weighed and homogenized in a Waring blendor for 30 seconds with 2 volumes of cold 0.1 M Na_2HPO_4 adjusted to approximately pH 7. Subsequent operations were carried out at 0–3°. The homogenate was centrifuged at 10,000 × g for 10 minutes and the precipitate was discarded.

A solution of ammonium sulfate, saturated at room temperature, which had been neutralized to pH 7 with NH_4OH, was added, with stirring, to the supernatant solution until it was 55 per cent saturated with ammonium sulfate. Stirring was continued for 20 minutes, following which the

mixture was centrifuged at 10,000 × *g* for 10 minutes. The precipitate was discarded, and the supernatant solution was made 70 per cent saturated with neutralized ammonium sulfate. After 20 minutes the mixture was again centrifuged and the precipitate was dissolved in 15 to 20 ml. of cold distilled water and dialyzed for 4 hours against 4 liters of distilled water. Any precipitate which formed during the dialysis was centrifuged and the protein concentration of the remaining solution was determined. The enzyme was treated with calcium phosphate gel (10) as follows: A gel suspension which had been aged for 18 months was centrifuged and the supernatant fluid was decanted. Enough gel was used so that the ratio of the protein to the dry weight of gel was 2:1. The enzyme solution was added to the sedimented gel and the mixture was stirred for 15 minutes and then centrifuged. The supernatant solution which contained all the steroid dehydrogenase activity represented approximately a 2-fold purification.

Ethanol, which had been chilled at −10°, was added dropwise to this solution until its concentration was 20 per cent. After 20 minutes, the resulting precipitate was removed by centrifugation at −5°. The supernatant solution was kept at −5°, and ethanol was again added until its concentration was 30 per cent. Again, after 20 minutes, the precipitate which formed was collected by centrifugation at −5° and dissolved in 15 ml. of distilled water. This solution was used for most of the experiments described. An additional 3-fold purification could be obtained if the enzyme was adsorbed on an equal weight of centrifuged calcium phosphate gel and eluted with 0.5 M phosphate buffer at pH 7.0. The results of this procedure were quite erratic, however, and it was not employed regularly. A summary of the purification procedure appears in Table II. It should be noted that there was a considerable increase in total activity following the first ammonium sulfate precipitation. When the crude extract was prepared with an all-glass homogenizer instead of a Waring blendor, no such increase in activity was observed. This was interpreted to mean that an inhibitor, probably the result of fragmentation of subcellular particles by the blendor blades, was removed by the first ammonium sulfate precipitation. No further study of this phenomenon was undertaken.

Properties of Enzyme

Stability—The first ammonium sulfate fraction was stable for at least 3 weeks at 3°, but its activity declined about 50 per cent in that time at −10°.

The most purified preparation lost between 80 and 100 per cent of its activity in 5 days stored at −10° and about 50 per cent of its activity at 3°.

The addition of DPN+ or dihydrocortisone did not retard this deterioration.

Effect of pH—The oxidation of DPNH by dihydrocortisone proceeds at a maximal rate between pH 7 and 8 in either Tris or phosphate buffer (Fig. 2).

TABLE II

Summary of Enzyme Purification

Fraction	Volume	Units	Specific activity*
	ml.		
Initial extract.....................	250	25,000	4.9
Ammonium sulfate ppt.............	40	74,500	64
Ca₃(PO₄)₂ gel extract..............	40	74,500	130
Ethanol (20–30%).................	15	30,000	375

* Units per mg. of protein.
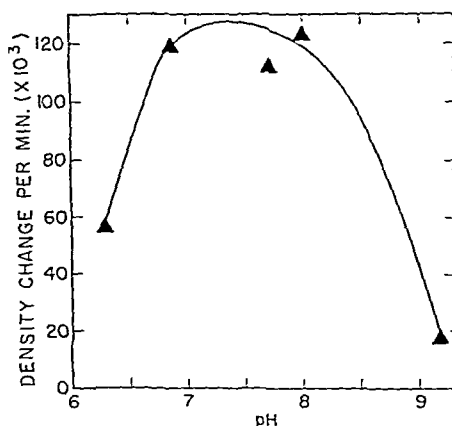
FIG. 2. Effect of pH on reaction velocity. The cuvettes contained 0.09 μmole of DPNH, 0.11 μmole of DHC, 20 μmoles of Tris buffer, and enzyme in a total volume of 1 ml.

The reduction of DPN by THC proceeds at a maximal rate between pH 8 and 8.3 but drops off rapidly above that value.

Substrate Affinity—The relation between reaction velocity and concentration of reactants is shown in Fig. 3. The K_m for dihydrocortisone is calculated to be 10^{-5} M, and that for DPNH is 1.5×10^{-5} M. TPNH purchased commercially or prepared enzymatically with glucose dehydrogenase had the same activity as DPNH. TPNH reduced by hydrosulfite, however, had no activity with any of the enzyme preparations tested.
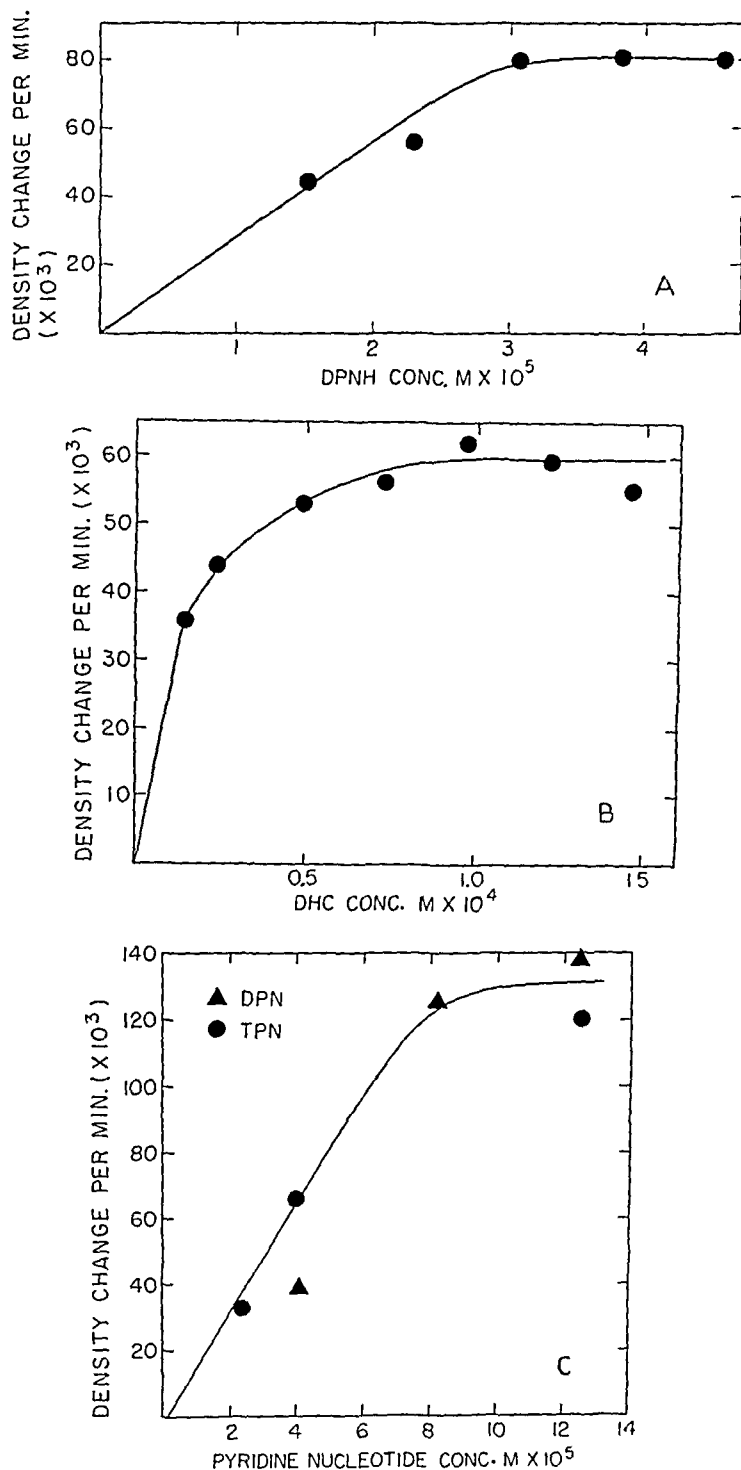
FIG. 3. Effect of concentration of reactants on reaction velocity. A, the cuvettes contained 0.05 μmole of DPNH, 20 μmoles of PO_4 buffer, pH 7.4, enzyme, and substrate in a total volume of 1.15 ml. B, the cuvettes contained 0.11 μmole of DHC, 20 μmoles of PO_4 buffer, pH 7.4, enzyme, and DPNH in a total volume of 1.15 ml. C, the cuvettes contained 0.11 μmole of THC, 20 μmoles of PO_4 buffer, pH 7.4, and pyridine nucleotide in a total volume of 1.15 ml.

In the reverse direction DPN+ and TPN+ function equally well. Fig. 3, *C* shows the reaction velocity as a function of oxidized pyridine nucleotide concentration. The K_m for DPN+ and TPN+ is 4×10^{-5} M.

Inhibition by Sulfhydryl Reagents—The presence of sulfhydryl groups in the enzyme was suggested by the inhibitory effect of heavy metals and

<div align="center">TABLE III</div>

<div align="center">*Inhibition by Sulfhydryl Reagents*</div>

The cuvettes contained 0.05 μmole of DPNH, 20 μmoles of PO₄ buffer, pH 7.4, 0.11 μmole of DHC, enzyme, and inhibitor to a volume of 1.2 ml. The enzyme was preincubated for 10 minutes at room temperature with buffer and the compounds noted below, and the reaction was started by the addition of the remaining components.

| Reagents preincubated with enzyme and buffer	Reagents initiating reaction	Per cent inhibition
Cu++, 10^{-4} M	DPNH, DHC	100
Iodoacetate, 10^{-4} M	" "	50
None	" " PCMB, 10^{-5} M	100
DHC	" PCMB, 10^{-5} M	46
DPNH	DHC, " 10^{-5} "	78
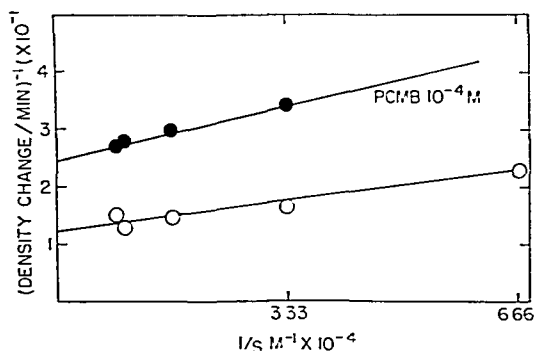
FIG. 4. Effect of PCMB on reaction velocity. The cuvettes contained 0.05 μmole of DPNH, 0.11 μmole of DHC, 20 μmoles of PO₄ buffer, pH 7.4, PCMB (or water), and enzyme in a total volume of 1.1 ml. O, no PCMB.

iodoacetate. The enzyme could be partially protected against PCMB inhibition by preincubating it with substrate or DPNH before the addition of PCMB (Table III). By the use of Lineweaver-Burk plots (11), PCMB was found to be a non-competitive inhibitor with respect to DHC (Fig. 4).

Determination of Equilibrium Constant—The value for the equilibrium constant, $K_{eq.} = ((THC)(DPN^+))/((DHC)(DPNH)(H^+))$, is calculated from a knowledge of the extent of DPNH oxidation and of the initial

concentrations of the reactants and is about 10^7. The data are presented in Table IV.

Substrate Specificity—3α-Hydroxysteroid dehydrogenase attacked both C_{19} and C_{21} 3-ketosteroids regardless of the configuration of the A-B ring

TABLE IV

Determination of Equilibrium Constant

The cuvettes contained 20 μmoles of phosphate buffer, enzyme, and the additions noted below in a volume of 1 ml.

Experiment No.	DPNH concentration, $\text{M} \times 10^3$		Initial DHC concentration, $\text{M} \times 10^3$	pH	K_{eq}.
	Initial	Final			
1	0.0725	0.050	0.081	7.8	1.12×10^7
2	0.045	0.008	0.184	7.0	1.16×10^7
3	0.027	0.007	0.073	7.0	1.08×10^7
Average..					1.12×10^7

TABLE V

Substrate Specificity

The cuvettes contained 0.05 μmole of DPNH, 0.11 μmole of substrate, 20 μmoles of phosphate buffer, pH 7.4, and enzyme to a volume of 1.15 ml.

Substrate	Relative rate of reduction
Dihydrocortisone.....................................	100
Pregnane-11β,17α,21-triol-3,20-dione.................	36
Etiocholane-3,17-dione..............................	110
Androstane-3,17-dione...............................	22
Androstan-17β-ol-3-one..............................	38
Cholestan-3-one.....................................	0
Coprostan-3-one.....................................	0
Cortisone...	0
Δ¹-Androstene-3,17-dione............................	0
Androstan-3β-ol-17-one..............................	0
Cyclohexanone......................................	0
Acetone..	0

junction, although A-B cis steroids were reduced more actively than their A-B trans isomers (Table V). The presence of the isooctyl side chain in cholestanone and coprostanone prevented the reaction, as did the presence of the α,β-unsaturation of cortisone and Δ¹-androstene-3,17-dione.

17-Hydroxy-3-ketosteroids did not oxidize DPNH and 3β-hydroxysteroids were not oxidized in the presence of the enzyme and DPN⁺. Cyclohexanone was not reduced by the purified preparation.

The data in Table VI suggest that a single enzyme catalyzes the reduction of both C_{19} and C_{21} steroids, since ratios of specific activities are relatively constant in preparations of differing purity. It should also be noted that

TABLE VI

Relative Activities (Units Per Mg. of Protein) of Different Enzyme Preparations with C_{21} and C_{19} Steroids As Substrate

Enzyme	DHC (A)	Etiocholane-3,17-dione (B)	$\frac{(B)}{(A)}$
Initial extract	13	14	1.1
Ammonium sulfate ppt	100	110	1.1
$Ca_3(PO_4)_2$ gel extract	225	225	1.0
Ethanol ppt	690	695	1.0
$Ca_3(PO_4)_2$ gel eluate	2000	2050	1.0

TABLE VII

Chromatography of Steroid Substrate and Reaction Products

Compound	Solvent system	Distance run for 16 hrs.
		cm.
DHC	Benzene-methanol-H_2O	15
THC	"	43
Reaction product of DHC	"	43
Pregnane-11β,17α,21-triol-3,20-dione	"	34
Pregnane-3α,11β,17α,21-tetrol-20-one	"	10
Reaction product of pregnane-11β,17α,21-triol-3,20-dione	"	10
Etiocholane-3,17-dione	Cyclohexane-methanol-H_2O	33.5
Etiocholan-3α-ol-17-one	"	20.5
Reaction product of etiocholane-3,17-dione	"	20.5
Androstane-3,17-dione	"	32.5
Androstan-3α-ol-17-one	"	24.5
Reaction product of androstane-3,17-dione	"	24.5

preparations, which had been partially inactivated by heat, dilute acid, or aging, retained relatively constant $C_{19}:C_{21}$ activity ratios.

Identification of Products of Reaction—For purposes of identification, 0.3 μmole of substrate, 0.5 μmole of DPNH, 800 γ of enzyme protein, and 20 μmoles of phosphate buffer were incubated in a total volume of 2 ml. at 38° for 30 minutes. The steroids were extracted with methylene chlo-

ride and subjected to paper chromatography according to a modification[3] of the Bush system (12).

The benzene-methanol-water mixture was used to separate and identify C_{21} substrates and metabolites. They were located on paper by means of the blue tetrazolium spray for reducing steroids (13).

The products of C_{19} steroid metabolism were chromatographed in the cyclohexane-methanol-water system and located by spraying the paper with the m-dinitrobenzene reagent of Zimmermann (5).

In every case, known compounds were chromatographed together with the products of the reaction. Their mobilities are presented in Table VII.

The enzymatic reduction of both the C_{19} and C_{21} 3-ketosteroids yielded only a single metabolite detectable by paper chromatography, and in each instance this had a mobility identical with that of the corresponding 3α-hydroxysteroid.

Further identification of the metabolic products was obtained by examination of their sulfuric acid chromogens (14) from 220 to 600 mμ. Following chromatography, the metabolites were eluted from paper with 95 per cent methanol. The methanol was evaporated under a stream of nitrogen, and the chromogens were developed. Known compounds, chromatographed and eluted in the same way, were also examined. The spectra of the reaction products so obtained were identical with those of the known corresponding 3α-hydroxysteroids which were expected as a result of the enzymatic reduction.

DISCUSSION

The presence of an enzyme (or enzymes) in mammalian tissue which catalyzes the interconversion of 3-keto- and 3α-hydroxysteroids has been inferred for some time on the basis of in vivo, slice, and perfusion studies (1). The data presented in this paper suggest that a single enzyme is responsible for these reactions regardless of whether the steroid is a C_{19} or a C_{21} compound, although it should be noted that the C_{27} 3-ketosteroids related to cholesterol are not metabolized by this preparation.

Because the liver steroid dehydrogenase described in this communication was found to contain thiol groups, the possibility was considered that a thiohemiketal was formed from enzyme-SH groups and the substrate carbonyl. Although no direct evidence for this was obtained, the fact that preincubation of the enzyme with DHC partially protects against PCMB inhibition suggests some substrate-SH interaction. Furthermore, it is of interest that Djerassi and Gorman (15) have synthesized cyclic thiohemiketals of steroid hormones at the 3 position.

The possibility that a single enzyme is involved in the reduction of a

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[3] Peterson, R. E., and Pierce, C. E., personal communication.

diverse group of steroids which have in common only a 3-keto group and which differ in physiological action suggests that, *in vivo*, these steroids might interact. Thus, the presence of one steroid substrate would impair the inactivation of another and thus enhance its activity.

SUMMARY

1. The partial purification of a 3α-hydroxysteroid dehydrogenase from mammalian liver has been described.

2. The enzyme catalyzes the interconversion of 3-keto- and 3α-hydroxysteroids of the C_{19} and C_{21} series, regardless of the configuration of A-B ring junction.

3. DPN^+ and TPN^+ can function equally well as hydrogen acceptors.

I should like to acknowledge the expert technical assistance of Miss Patricia J. Michael during the course of this work.

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DETOXICATION OF CYANIDE BY CYSTINE*

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The reaction of cyanide with cystine yields cysteine and β-thiocyano-alanine (I). Schöberl, Kawohl, and Hamm (1) have shown that the latter tautomerizes to 2-aminothiazoline-4-carboxylic acid (II) or the equivalent 2-imino-4-thiazolidinecarboxylic acid (III).
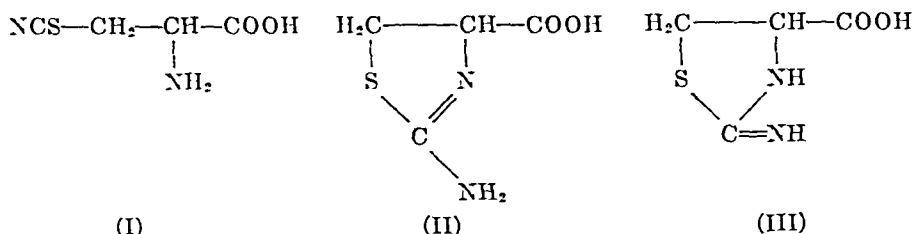
The chemical properties of this cyanide-cystine reaction product have been studied by Schöberl and Hamm (2), Aldrich (3), and Behringer and Zillikens (4). Further characterization of the compound and its chemical reactions have been investigated in this laboratory. Particularly, possible rôles in the reactions of intermediary metabolism were sought. It was found that the intact animal could convert the compound neither to cysteine nor to thiocyanate. During these studies, it became apparent that 2-imino-4-thiazolidinecarboxylic acid was inert metabolically when administered to the rat. When cyanide entered the body, 2-imino-4-thiazolidinecarboxylic acid was formed *in vivo* and was excreted in the urine. This reaction constitutes a new, independent pathway for detoxication of cyanide.

EXPERIMENTAL

2-Imino-4-thiazolidinecarboxylic Acid—The procedure of Schöberl and Hamm (2) was followed essentially, except that the product was isolated in crystalline form from 70 per cent ethanol without water of hydration. The yield was 66 per cent of theory. After recrystallization, the compound decomposed at 212° and had a specific rotation of $[\alpha]_D^{29}$ −2.08° (1 per cent in water).

$C_4H_6O_2N_2S$ (146.2). Calculated, S 21.8, N 19.2; found, S 21.3, N 19.1

The compound showed only end-absorption in the ultraviolet region of the spectrum. Infra-red absorption spectra on the solid mulled in Nujol were in general consistent with that of the ring structures, but furnished no distinction between the two tautomers.[1] Two sharp bands in the 3400 cm.$^{-1}$ region, which are characteristic of stretching vibrations of unassociated NH_2 groups of 2-aminothiazoline, were absent in the cystine derivative, owing possibly to association with the carboxyl. A band at about 1640 cm.$^{-1}$, characteristic of stretching vibrations for cyclic $C\!=\!N$ groups or for NH_2 deformation vibrations, was present in 2-aminothiazoline and the 4-carboxylic acid derivative prepared from cystine. The 3-acetyl derivative, which necessarily had the iminothiazolidinecarboxylic acid structure, also had strong bands at 1650 and 3290 cm.$^{-1}$.

A 0.1 mmole sample of L-cystine was suspended in 25 ml. of potassium phosphate buffer, pH 7.4. Amperometric sulfhydryl determinations by the method of Weissman, Schoenbach, and Armistead (5) were used to follow the course of the reaction of the cystine with 0.5 gm. of potassium cyanide. The production of free sulfhydryl at 5, 10, and 20 minutes was 42, 80, and 100 per cent of the theoretical value, respectively.

The iminothiazolidine was converted to alanine by treatment with Raney nickel as described by Behringer and Zillikens (4). The 2-imino-4-thiazolidinecarboxylic acid, its ester, 3-acetyl derivative, and corresponding ester each gave a cherry-red color when incubated with diazotized sulfanilic acid (1). An excess of the substance under test interfered with the reaction. On paper chromatograms, 5 γ of the iminothiazolidine could be detected by this color reaction.

3-Acetyl-2-imino-4-thiazolidinecarboxylic Acid—The 2-iminothiazolidinecarboxylic acid was acetylated with acetic anhydride in aqueous alkaline solution according to the method of du Vigneaud and Irish (6). The yield of colorless crystals was essentially quantitative. The product, after crystallization from ethanol and then from chloroform, melted at 179–180° and had an optical rotation of $[\alpha]_D^{29}$ −1.52° for a 1 per cent solution in water.

$C_6H_8O_3N_2S$ (188.2). Calculated, S 17.0, N 14.9; found, S 16.8, N 15.0

Hydrolysis of a 10 mg. sample with dilute sulfuric acid at 100° yielded the original iminothiazolidine and some thiocyanate which was identified by paper chromatography.

3-Acetyl-2-imino-4-thiazolidinecarboxylic acid was also prepared from N,N'-diacetylcystine. Cystine (1.5 gm.) was acetylated according to the method of Hollander and du Vigneaud (7). Paper chromatograms demonstrated the absence of sulfur-containing impurities in the preparation. The

[1] We are indebted to Dr. Nelson Fuson of Fisk University and Mr. H. G. McDonnell, Jr., of The Perkin-Elmer Corporation for infra-red spectrograms.

diacetylcystine was dissolved in water and treated with 0.5 gm. of potassium cyanide. After standing at room temperature for 30 minutes, the solution was treated with 10 per cent hydrogen peroxide until the nitroprusside test for SH was negative. The procedure was repeated when, 30 minutes later, a positive nitroprusside test had developed. The solution was acidified to pH 5 and concentrated to dryness *in vacuo*. The residue was taken up in chloroform, which was evaporated, and a crystalline residue, m.p. 180°, was obtained. This was identified as the acetyl derivative by comparison on paper chromatograms and by a melting point determination on a mixture of the sample with an authentic specimen of 3-acetyl-2-imino-4-thiazolidinecarboxylic acid.

Ethyl 2-Imino-4-thiazolidinecarboxylate Hydrochloride—2-Imino-4-thiazolidinecarboxylic acid (5 gm.) was dissolved in ethanol, and the solution was saturated with dry hydrogen chloride. After 24 hours, an equal volume of ether was added. Crystalline ethyl ester hydrochloride separated. The yield was 5 gm. After recrystallization from ethanol the product melted at 115–116°.

$C_6H_{11}O_2N_2SCl$ (210.2). Calculated, S 15.2, N 13.3; found, S 15.0, N 13.1

Ethyl 3-Acetyl-2-imino-4-thiazolidinecarboxylate—This was prepared by esterifying 5 gm. of the acetyl derivative with ethanol and dry hydrogen chloride as described above. The product weighed 3.5 gm., and after recrystallization from ethanol melted at 136°.

$C_8H_{12}O_3NS$ (216.3). Calculated, S 14.8, N 12.0; found, S 14.7, N 12.8

Metabolism Studies

2-Imino-4-thiazolidinecarboxylic acid, its ethyl ester, 3-acetyl, and 3-acetyl ethyl ester derivatives were tested as substrates for rhodanese by the method of Himwich and Saunders (8). None of these compounds yielded thiocyanate when incubated with rhodanese. Inhibition studies at levels of 0.042 and 0.42 M concentration were also made. The compounds were without effect as inhibitors on the formation of thiocyanate from thiosulfate.

10 mmoles of each iminothiazolidine derivative were incubated anaerobically for 2 hours with 10 ml. of Krebs-Ringer bicarbonate buffer, 2 mg. of glutathione, and 2 gm. of rat liver slices. Determinations on the supernatant solution and homogenized slices revealed no thiocyanate formation. Repetition of the incubation under 95 per cent oxygen-5 per cent carbon dioxide gas mixture also did not produce any thiocyanate. Rat liver homogenates were likewise ineffective.

Preliminary studies were made on dietary replacement of cystine by 2-imino-4-thiazolidinecarboxylic acid. Two young rats growing slowly on a diet consisting of 18 per cent desulfurized casein (9) plus 0.13 per cent

DL-methionine failed to respond to incorporation of 0.2 per cent 2-imino-4-thiazolidinecarboxylic acid in the food. A corresponding lack of effect was shown by 0.2 per cent of the compound when added to the 8 per cent casein diet of two rats. Further work along this line was abandoned when the metabolic inertness of the compound was developed in the following experiment.

2 gm. of imino-4-thiazolidinecarboxylic acid mixed with 20 gm. of 20 per cent casein diet were consumed by an adult male rat in 3 days. The urine was collected in a tube cooled by dry ice. The compound fed was isolated by precipitation with Barfoed's reagent, followed by treatment of the precipitate with H₂S. The recovery from the urine was 300 mg. of crystalline material, which was identified by paper chromatography. None of the acetyl derivative could be found in the urine.

Three young rats were fed diets containing from 2.5 to 5 per cent 2-imino-4-thiazolidinecarboxylic acid for periods of from 8 to 10 days. In each case the urines were collected and examined for evidence of the acetyl derivative by continuous extraction from acidified urine with chloroform. None of the acetyl derivative was found, but the original compound fed was isolated from all the samples by precipitation as the copper salt. It was observed that when acid solutions were subjected to paper chromatography the hydrochloride of 2-imino-4-thiazolidinecarboxylic acid migrated approximately as the 3-acetyl derivative.

Injection Studies

A 250 gm. male rat was injected intraperitoneally with 20 mg. of S³⁵-labeled 2-imino-4-thiazolidinecarboxylic acid dissolved in 0.6 ml. of sodium bicarbonate solution. The animals were maintained on a 10 per cent casein diet. Urine was collected for 3 day periods. The receiver contained a few drops of concentrated hydrochloric acid. Paper chromatograms showed two radioactive spots corresponding to 2-imino-4-thiazolidinecarboxylic acid and thiocyanate. The latter, however, proved to be an artifact caused by the acid. A sample of urine containing the 2-iminothiazolidine but no thiocyanate was acidified to Congo red with HCl and allowed to stand at room temperature for 3 days. Subsequent chromatograms showed the presence of radioactive thiocyanate ion. The excretion of injected labeled 2-imino-4-thiazolidinecarboxylic acid as measured by radioactivity determinations on the urine and feces when subjected to paper chromatography is reported in Table I. In the second and third instances, the urines were collected in a tube cooled with dry ice. Such samples showed no radioactivity at the thiocyanate spot. In the third experiment, in order to increase the sensitivity of detection, the animal was "washed out" by injection of 5 mg. of potassium thiocyanate on the 2nd day.

Formation of 2-Imino-4-thiazolidinecarboxylic Acid in Vivo—A male white rat weighing 210 gm. was injected by tail vein with 20 mg. of L-cystine-S^{35} having a measured radioactivity of 10^7 c.p.m. After 15 minutes had elapsed, 1 mg. of sodium cyanide was injected subcutaneously, and 10 minutes later an additional 1 mg. of cyanide was administered. The animal was placed in a metabolism cage and given a 10 per cent casein diet. The

TABLE I

Excretion of Injected 2-Imino-4-thiazolidinecarboxylic Acid-S^{35}

Weight of rat	Amount injected	Per cent recovered			
		1–3 day urine	4–6 day urine	7–11 day urine	Total feces
gm.	*mg.*				
250	20	79.8	7.78	4.25	
250	25	98.5	1.00		0.3
200	35	81.2	5.06	4.56	1.1

TABLE II

Distribution of Radioactive Sulfur in Urine of Rat after Injection of Cystine-S^{35} and Cyanide

Radioactive component on paper segment	1st day	2nd day	3rd day	Amount of excreted S^{35} in 3 days*
	γ	γ	γ	*per cent*
Thiocyanate.....................	10.5	9.8	0	1.7
2-Imino-4-thiazolidinecarboxylic acid......	229	42.6	0	23
S^{35} compounds diffusely distributed†	136	0	0	11.7
Compounds at origin‡	500	187	68	63.3

* A total of 5.3 mg. of cystine-S^{35} sulfur was injected.

† 136 γ of S were diffusely distributed between the origin and the 2-iminothiazolidine acid segment.

‡ Includes sulfate, cystine, and other salts which do not migrate under these conditions.

urine was collected in a tube cooled in a dry ice-ethanol bath. Three 24 hour samples were collected, melted, filtered through glass wool, and diluted to 10 ml. 75 μl. aliquots of the urine were chromatographed on paper with 77 per cent ethanol as a solvent. Sulfate, cystine, and methionine were not resolved by this procedure. Radioactivity measurements made directly on segments of the chromatogram were corrected for self-absorption of the paper. The results are presented in Table II. A 0.1 ml. aliquot of the 1st day's urine was chromatographed in a 12 inch band on filter paper. The position of the 2-imino-4-thiazolidinecarboxylic acid was determined

by spraying a 1 inch strip with iodine-sodium azide reagent. A 10 inch strip was cut into segments, and the 2-imino-4-thiazolidinecarboxylic acid section was eluted with water. To the eluate were added 10 mg. of 2-imino-4-thiazolidinecarboxylic acid, and the sample was recrystallized three times. A test for radioactive impurities was made by the solubility product method of Gutmann and Wood (10). This showed that radioactivity was not due to an impurity in the isolated crystals and hence substantiated the presence of radioactive detoxication products in the urine.

A female rat weighing 310 gm. was placed in a metabolism cage which was arranged so that the urine would be collected in a tube cooled with dry ice-ethanol freezing mixture. A 10 per cent casein diet was supplied *ad libitum*. Several daily intraperitoneal injections of 1.5 mg. of potassium cyanide in 1 ml. of water were made over a period of 8 days. The animal received a total of 28.6 mg. (0.44 mmole). The urine samples were pooled and evaporated *in vacuo* to a volume of 5 ml. Nine-tenths of the urine was treated with Barfoed's reagent, and the iminothiazolidine was isolated without dilution as described previously. The product was obtained as 10 mg. of crystalline material (0.068 mmole). A recrystallized portion had a melting point of 212°. The compound was identified as 2-imino-4-thiazolidinecarboxylic acid by comparison with an authentic sample and by paper chromatography with sulfanilic acid and iodine-azide reagents. A determination of the thiocyanate content was made on an aliquot of the urine. A total of 0.35 mmole (80 per cent as much as the cyanide injected) of thiocyanate was found.

In a repetition of the experiment, 35 mg. of potassium cyanide were injected into a 310 gm. male rat over a period of 6 days. 2-Imino-4-thiazolidinecarboxylic acid was isolated from the urine in the manner described above. Thiocyanate was also detected by its color reaction with ferric ion.

It had been found previously that the thiocyanate content of the saliva of workers was elevated following extensive work with cyanide solutions. In a particular instance in our laboratory, a worker had experienced an exceptionally long exposure to sublethal amounts of cyanide. A sample of saliva was collected and treated with 9 volumes of ethanol. The supernatant fluid was concentrated to a small volume, and a sample equivalent of 1 ml. of original saliva was chromatographed on filter paper with 77 per cent ethanol as the developing solvent. When the chromatogram was sprayed with iodine-azide reagent, sulfur-containing compounds were noted at the positions occupied by thiocyanate and 2-imino-4-thiazolidinecarboxylic acid. A second chromatogram was sprayed with diazotized sulfanilic acid solution and revealed a red spot at the position occupied by 2-imino-4-thiazolidinecarboxylic acid. 2 weeks later none of this compound or thiocyanate was present in the saliva of the same subject by the same tests. A sample of this saliva was then treated with cyanide *in vitro*

for 30 minutes. Subsequent chromatograms showed the presence of thiocyanate and 2-imino-4-thiazolidinecarboxylic acid.

DISCUSSION

The structural nature of the cyanide-cystine reaction product had considerable bearing on predictions about its physiological behavior. Thus the open chain form, β-thiocyanoalanine, should be a source of thiocyanate in the body (11), since it could undergo oxidative deamination to produce thiocyanopyruvic acid which has been shown in this laboratory to decompose readily to yield thiocyanate ion (1). Furthermore, Aldrich (3) synthesized a product from cysteine and cyanogen chloride which was evidently the same as the cyanide-cystine reaction product. Aldrich also made an acetyl derivative which reacted with sulfhydryl compounds to yield free cyanide ion and presumably acetylcysteine. In the present studies, the cyanide-cystine reaction product was not acetylated by the animal body. Hence it would not be expected to undergo these changes *in vivo*.

Neither the cyanide-cystine product nor its derivatives yielded thiocyanate under physiological conditions. The apparent excretion of thiocyanate after injection of 2-imino-4-thiazolidinecarboxylic acid was shown to be an artifact resulting from decomposition of the compound on standing in acidified urine at room temperature.

From the work of Nicolet (12) on the lability of cysteine derivatives, the formation of thiocyanate, pyruvic acid, and ammonia might be predicted if appreciable amounts of the open chain tautomer, β-thiocyanoalanine, occurred in alkaline solution. This was not realized experimentally, owing no doubt to the rapid formation of cysteine hydantoin as found by Schöberl and Hamm (2).

The above observations suggest that in the physiological range of hydrogen ion concentration significant quantities of the open chain form, β-thiocyanoalanine, do not exist in equilibrium with the ring forms. Schöberl, Kawohl, and Hamm concluded, from their chemical studies, that the reaction product of cystine and cyanide was a cyclic structure. The present studies support this conclusion with somewhat different evidence. In particular, no reaction characteristic of organic thiocyano compounds could be demonstrated. Infra-red spectra supported the cyclic structures for the compound in the solid state without differentiating between the aminothiazolidine and iminothiazolidine forms. The acetylation product was indicated as the iminothiazolidine form by its synthesis from N,N'-diacetylcystine and cyanide.

The rapidity of the cyanide-cystine reaction suggested that appreciable amounts of the thiazolidinecarboxylic acid should form in the blood. Since the compound is readily excreted, it qualifies as a detoxication product of cyanide. Preliminary qualitative studies have revealed the presence

of traces of the product in the urine of individuals experiencing industrial exposure to hydrogen cyanide.   It is likely that the thiazolidine derivative has not been identified previously on chromatograms of urine and saliva because of its failure to produce a purple spot with ninhydrin (1).   At present there is no evidence for enzymatic catalysis; the reaction is sufficiently rapid for production of the product in the saliva *in situ.*

Voegtlin, Johnson, and Dyer (13) showed that cystine protected animals from minimal lethal doses of cyanide.   The effect was obtained by injecting cystine into the blood stream immediately before the subcutaneous injection of cyanide.   Present interpretation of these studies accounts for the protective effect by the formation of 2-imino-4-thiazolidinecarboxylic acid from a relatively high concentration of free cystine in the blood.   Using cystine-S^{35}, we found that the injected labeled amino acid produced about 13 times as much 2-imino-4-thiazolidinecarboxylic acid as thiocyanate. Since cystine does not serve as a substrate for rhodanese directly, the labeled thiocyanate excreted was produced subsequent to metabolic alteration of the injected cystine.   Iminothiazolidinecarboxylic acid formation is small compared with other pathways of detoxication.   In an experiment in which no cystine was injected, the recovery of intraperitoneally injected cyanide as thiocyanate and as the thiazolidine was 80 and 15 per cent, respectively. The prophylactic action of cystine against cyanide poisoning is a direct detoxication reaction, apparently potentiated by raising the blood level of the sulfur amino acid.

SUMMARY

Chemical studies on the nature of the reaction product between cystine and cyanide ion support formulation of the structure as 2-imino-4-thiazolidinecarboxylic acid.   The compound was inert metabolically when fed to the rat or when injected.   On treatment with acid, a small amount of thiocyanate was produced.

2-Imino-4-thiazolidinecarboxylic acid was isolated from the urine of rats given sodium cyanide subcutaneously.   80 per cent of the cyanide was accounted for as thiocyanate.   When L-cystine-S^{35} was administered first, the compounds excreted were labeled.   Radioactivity measurements showed that the iminothiazolidinecarboxylic acid was produced from cystine, while the thiocyanate chiefly was formed from other sources of sulfur.

The reaction with cystine constitutes an independent pathway for detoxication of cyanide.

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THE RELATIONSHIP OF EPINEPHRINE AND GLUCAGON TO LIVER PHOSPHORYLASE

I. LIVER PHOSPHORYLASE; PREPARATION AND PROPERTIES*

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The concentration of phosphorylase in liver slices changes rapidly in response to certain experimental conditions (1–4). The concentration of phosphorylase in the cells represents a balance between inactivation of the enzyme and reactivation to the active form. Inactivation of liver phosphorylase is catalyzed by an enzyme formerly designated liver phosphorylase-inactivating enzyme. The synthetic aspect of the balance, *i.e.* the reactivation of the inactive liver phosphorylase, is influenced by epinephrine and glucagon so that resynthesis of the active form is promoted (5). The experiments reported in the following papers were designed to aid our understanding of the mechanisms involved in the regulation of the concentration of liver phosphorylase in intact cells. Liver phosphorylase and the enzyme from liver which inactivates it have been prepared in purified form. The enzymatic inactivation of liver phosphorylase has been studied (6) and also the process of reactivation in slices and extracts (5).

This report deals with the preparation and properties of liver phosphorylase. Two major problems required solution. The enzymatic inactivation of liver phosphorylase proceeds rapidly unless precautions are taken. Consequently, the early steps have been designed primarily to inhibit, denature, or remove the liver phosphorylase-inactivating enzyme. The second problem was created by the tendency of liver phosphorylase to accompany glycogen during fractionation. As purification proceeded, the ratio of carbohydrate to protein increased to values of 20 or 30 to 1, and, therefore, steps were taken to lower the glycogen concentration of the preparation. The procedures described here have resulted in greater yields of enzyme with a higher specific activity than reported previously, even though apparently homogeneous preparations of liver phosphorylase were obtained by an earlier procedure (7).

Methods

Materials—Glycogen, G-1-P,[1] and 5-AMP were purchased from the Nutritional Biochemicals Corporation.

G-1-P was purified by the following procedures. 2 liters of a 5 per cent solution were chilled, 3 gm. of Norit were added, and the pH was adjusted to 3.5 with glacial acetic acid. The solution was brought to a concentration of 50 per cent ethanol, filtered, and adjusted to pH 8.0 with KOH (as measured with a glass electrode), additional ethanol being added to maintain a 50 per cent concentration. After storage at 3° overnight, the crystals were collected and dissolved in 1.2 liters of glass-distilled water and filtered at room temperature. The filtrate was again brought to a concentration of 50 per cent ethanol. After storage at 0° overnight, the crystals were collected by decantation and centrifugation and washed successively with 95 per cent ethanol, absolute ethanol, absolute ethanol and absolute ether (1:1), and absolute ether. The yield of dry product was usually about 75 per cent of the starting material.

Glycogen was purified in the following manner before use. A 5 per cent solution was made and filtered and, if yellow, decolorized with Norit. The solution was then brought to a concentration of 50 per cent ethanol and allowed to stand overnight at 3°. The centrifuged precipitate was washed with absolute ethanol, absolute ethanol and absolute ether (1:1), absolute ether, and dried.

Tris, obtained from the G. Frederick Smith Chemical Company, was purified in the following manner. 400 gm. of Tris were dissolved in 2 liters of 85 per cent ethanol containing 0.6 gm. of trisodium Versene at 73°. The solution was filtered at 70° and chilled overnight. The supernatant solution was decanted and the crystals were collected on a Büchner funnel and washed in the same fashion as for G-1-P. The washed crystals were stored at room temperature in an evacuated desiccator (over $CaCl_2$) for 2 days before use. The yield was usually 80 per cent.

Merck reagent grade ammonium sulfate was recrystallized in the presence of Versene in the following fashion. 3 liters of distilled water containing 3 gm. of disodium Versene and 3 ml. of concentrated ammonium hydroxide were saturated with ammonium sulfate at about 70°. The solution was filtered rapidly through Whatman No. 50 filter paper and the filtrate was allowed to stand overnight at 3°. The crystals were removed and more ammonium sulfate was precipitated by the addition of ethanol to about 10 per cent concentration and by allowing the mixture to stand overnight. All crystalline material was washed successively on a Büchner funnel with ethanol of 30, 50, 95, and 100 per cent concentration. The washed crystals

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[1] The following abbreviations are used: G-1-P, glucose-1-phosphate; 5-AMP, adenosine-5-phosphate; Tris, tris(hydroxymethyl)aminomethane.

were stored in an evacuated desiccator for 24 hours before use. The yields were about 20 per cent.

The calcium phosphate gel was prepared essentially as described (8), distilled water being employed at all times. The final preparation contained about 21 mg. of calcium phosphate per ml.

Standard Assay—Phosphorylase activity was determined by measurement of the rate of liberation of inorganic phosphate from G-1-P in the presence of glycogen; *i.e.*, phosphorylase activity was measured in the direction of polysaccharide synthesis. A stock solution of G-1-P (0.036 M adjusted to pH 6.1 with HCl) containing 4.03 mg. of glycogen per ml. and 0.1 M NaF was stable in the frozen state and when thawed constituted the basic reagent for assay. This reagent was usually prepared to contain 1.4×10^{-3} M 5-AMP. Enzyme dilutions were made immediately before assay in cold 0.1 M NaF. The reaction was started by the addition of 0.2 ml. of enzyme to 2.8 ml. of the basic assay reagent at room temperature. Aliquots of the reaction mixture (0.5 ml.) were transferred to trichloroacetic acid at zero time and after incubation for 10 minutes at 37°; the remainder was used for an iodine starch test.[2] The inorganic phosphate present in an equivalent of 0.1 ml. of reaction mixture was determined by the method of Fiske and Subbarow (9) as adapted to the Klett-Summerson photometer. In crude preparations small corrections can be made for phosphatase activity by determination of glucose production due to the addition of G-1-P.

1 unit of enzyme was defined as that amount which caused the liberation of 1.0 mg. of inorganic phosphorus in 10 minutes when the per cent conversion of glucose-1-phosphate was in the range of 12 to 22 per cent. Specific activity was expressed as units per mg. of protein. Protein was determined by a micromethod described previously (10), modified according to the procedure of Lowry *et al.* (11).

The purified enzyme may be assayed more accurately at pH 6.7 with a final glycogen concentration of 1.0 per cent and 1.6×10^{-2} M G-1-P. Under these conditions at 30° first order kinetics are followed, as described by Cori *et al.* (12).

Results

Step 1. Preparation of Filtrate—Medium sized or large dogs of various mixed breeds were obtained from the pound and were used approximately 20 hours after the last feeding. 2 ml. of a 1:1000 epinephrine solution were

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[2] The iodine starch test was valuable for rapid estimation of activity and as evidence that polysaccharide formation accompanied the formation of inorganic phosphate. This additional routine test could not be used when the reaction mixture contained amounts of glycogen sufficient to yield first order kinetics. Therefore, the above assay was adopted even though first order kinetics were not obtained.

given intraperitoneally 5 to 10 minutes before a lethal dose of sodium seco-barbital was injected intravenously. The carotid vessels were severed and the thorax and abdomen were opened. A large cannula was inserted into the portal vein and the inferior vena cava was cut just below the heart. Several liters of cold sodium fluoride (0.2 M) were perfused through the liver; intermittent pressure on the inferior vena cava just above the diaphragm increased the filling of the liver and the effectiveness of the perfusion. At the start of the perfusion, 3 ml. of 1:1000 epinephrine were in-jected into the rubber perfusion tubing leading to the cannula. The chilled perfused liver was removed, and, after separation of the gallbladder, was immersed in chilled 0.1 M NaF. The liver was divided into 100 gm. portions, each of which was cut into small pieces and homogenized in a Waring blendor for 2.5 minutes in 400 ml. of cold 0.1 M NaF containing 0.005 M K_2HPO_4. These and all subsequent steps were carried out at 3° unless specified otherwise. The pooled homogenates were adjusted to pH 5.7 with 1.0 N acetic acid. An amount of Johns-Manville Hyflo Super-Cel equal to the weight of the liver was mixed with the homogenate and the mixture was filtered under 20 pounds pressure by using a No. 0 pad in a Hormann filter press.[3] The cake was washed by the addition of cold 0.1 M NaF to the top of the cake, the volume of the wash fluid equaling 1.2 times the weight of the liver.

Step 2. Adsorption and Ammonium Sulfate—The turbid tan or pink filtrate was adjusted to pH 6.5 with 1.0 N KOH. Part of the phosphorylase-inactivating enzyme was removed by adsorption on calcium phosphate gel. $\frac{1}{12}$ volume of gel was added to the filtrate and stirred occasionally for 15 minutes before centrifugation. (Further purification of the inactivating enzyme is described in Paper II (6).) The supernatant fluid from the gel was adjusted to pH 7.2 with 1 N KOH and the 0.0 to 0.66 ammonium sulfate fraction was precipitated by the addition of 46 gm. of solid ammonium sulfate per 100 ml. of solution. The pH was adjusted to 7.2 again with 1.0 N KOH and, after standing for 15 minutes, the fraction was collected by centrifugation for 20 minutes at 7000 × g. The supernatant fluid was discarded and a second precipitate was collected in the same tubes above the first precipitate. The precipitates were dissolved in 0.1 M NaF, 75 ml. for each 100 gm. of liver, and 10 ml. of 0.02 M 5-AMP per 100 gm. of liver were also added. 5-AMP protected liver phosphorylase against enzymatic inactivation and heat denaturation in the next step. The pH was adjusted to 7.1 with 1.0 N KOH and the preparation was ready for the heat denaturation step or, alternatively, it could be stored in the frozen state. The final volume of the solution was measured and the increase in volume above the

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[3] Obtained from F. R. Hormann and Company, Inc., 17 Stone Street, Newark 4, New Jersey.

volume of added solutions was taken as a measure of the amount of ammonium sulfate present as 0.66 saturated solution.

Step 3. Heat Denaturation of Inactivating Enzyme—The preparation was transferred to a 2 liter Erlenmeyer flask and washed in with 30 ml. of 0.1 M NaF, thus giving a final concentration of about 0.17 saturated ammonium sulfate. It was then heated with swirling in a bath at 67° until the temperature rose to 55°. The temperature was maintained at 55° for 5 minutes; the solution was then chilled in an ice water bath. The preparation was centrifuged for 20 minutes at $7000 \times g$, and the precipitate was discarded.

Step 4. Collection of 0.41 to 0.8 Ammonium Sulfate Fraction—Heated extracts from the livers of three dogs were pooled at this stage to minimize variations in fractionation. The concentration of ammonium sulfate was calculated and then a neutralized ammonium sulfate solution saturated at room temperature was added to bring the preparation to 0.41 saturation. The measured specific gravity at this calculated saturation was 1.13 at 3°. The precipitate obtained by centrifugation at $7000 \times g$ for 20 minutes contained an amount of phosphorylase which varied with the glycogen content of the preparation. The 0.41 fractions obtained from extracts containing moderate or large amounts of glycogen were discarded, since most of the phosphorylase remained in the supernatant fluid. Removal of the 0.41 fraction did not cause any substantial increase in specific activity, but was carried out to eliminate protein which would otherwise precipitate with alcohol in subsequent steps. The 0.41 to 0.8 fraction was obtained by adding solid ammonium sulfate to the supernatant solution of the 0.41 precipitate (27.3 gm. per 100 ml. of supernatant solution), followed by centrifugation at $7000 \times g$ for 20 minutes. Precipitates from two centrifugations were collected in the same tubes; the 0.8 supernatant fluid was discarded. It was found that the 0.41 to 0.8 fraction was stable in the cold overnight; hence centrifugation could be carried out later. The 0.41 to 0.8 precipitates were dissolved by the addition of 170 ml. of cold 0.1 M NaF per 1000 gm. of liver. This solution could be frozen at −20° and kept for some days without serious loss of activity.

Step 5. Dialysis of 0.41 to 0.8 Ammonium Sulfate Fraction and Ethanol Fractionation—The ammonium sulfate concentration of the preparation was lowered before alcohol fractionation to avoid denaturation of phosphorylase. Since traces of inactivating enzyme sometimes remained, the dialysis was carried out in the cold with fluoride for periods no longer than indicated. Dialysis with continued agitation was carried out in Visking casing (size 27/32) *versus* 10 volumes of NaF (0.1 M) containing 5×10^{-4} N KOH for 1 hour; then the dialysis was continued for another hour *versus* a fresh NaF-KOH solution. After dialysis, 0.1 volume of 5-AMP (0.02 M, pH 7.0)

was added and the pH of the solution was adjusted to approximately 7.0 with KOH. The preparation was then ready for ethanol fractionation but could be kept frozen at −20°. To the preparation, chilled in an ice bath, cold (−20°) ethanol was added slowly with stirring until a 25 per cent final concentration was reached; then the preparation was chilled to 3° and centrifuged. The supernatant fluid was discarded, the precipitate was dissolved in 0.1 M NaF (two-thirds of the volume before adding ethanol), and 0.02 M 5-AMP was added (10 per cent of the volume of the added NaF). Alcohol was again added to a final concentration of 25 per cent; the precipitate was collected as previously and redissolved and reprecipitated as above

TABLE I

Summary of Purification and Yield; Step 1 through Step 5

Livers from three dogs were fractionated separately through Step 3, then pooled for Steps 4 and 5. The specific activity of the homogenate was about one-third that of the filtrate. No corrections have been made for sampling nor for loss in precipitates.

Fraction	Total units	Specific activity*	Per cent original activity
Step 1. Filtrate	25,200	0.31	100
" 2. 0.0–0.66 ammonium sulfate	22,786	0.44	90
" 3. Heated 0.0–0.66 supernatant fluid	12,491	0.46	50
" 4. 0.41–0.80 ammonium sulfate	11,400	0.57	45
" 5. 0.41–0.80 after dialysis	10,300	0.55	41
1st alcohol	9,600	4.15	38
2nd "	8,900	5.7	35
3rd "	7,650	6.2	30

* See the text for unit of measurement.

to obtain the third alcohol precipitate. Occasionally the alcohol precipitation was repeated one or more times until the supernatant solution was colorless. The third alcohol precipitate from 1000 gm. of liver was dissolved in 200 ml. of water and appeared milky because of the large amounts of glycogen present. If the precipitate was very bulky, 300 ml. of water were used. The third alcohol fraction was stable in the frozen state for several weeks or months. The results of a typical fractionation to this stage are summarized in Table I.

Step 6. Adsorption and Elution—This step was designed to free the preparation of most of the glycogen which accompanied the preceding fractions in large amounts. The third alcohol fraction was diluted with cold glass-distilled water so that it contained approximately 3 units per ml. Usually an 8-fold dilution was made and pilot experiments were carried out to de-

termine the amount of calcium phosphate gel necessary for nearly complete adsorption of the enzyme. (Usually 2 to 3 per cent of the volume of the diluted, third alcohol fraction was sufficient.) Calcium phosphate gel was added to the diluted, cold, third alcohol fraction and stirred occasionally for 15 minutes. The gel was collected by centrifugation and washed with cold 0.001 M Tris, pH 7.4, which had been recrystallized in the presence of Versene, the volume of the wash fluid equaling the discarded supernatant solution. The gel was again collected by centrifugation and eluted for 10 minutes at 25° with 0.01 M potassium citrate (pH 6.5), the volume of citrate equaling 12 per cent of the diluted third alcohol volume.[4] The eluted gel was packed by centrifugation at room temperature for 10 minutes at 7000 × g and discarded.

Step 7. Fourth Alcohol Collection and Dialysis Versus Phosphate—The eluate, containing the phosphorylase, was chilled and to each 100 ml. were added 0.55 gm. of NaCl and 40 ml. of absolute ethanol (−20°). The mixture was chilled to 3° and centrifuged. The precipitate was dissolved or suspended in 0.1 M potassium phosphate (pH 7.2) with approximately 20 ml. per 1000 gm. of liver. The suspended precipitate was placed in washed Visking casing (size 27/32) and dialyzed *versus* 3.5 liters of 0.1 M potassium phosphate (pH 7.2) for 16 hours at 22°. During this dialysis the small amounts of glycogen remaining were partially digested and a heavy, flocculent precipitate was formed. The inactive flocculent precipitate was removed by centrifugation for 15 minutes at 7000 × g at room temperature and discarded.

Step 8. Second Adsorption and Elution—The dialyzed and centrifuged fourth alcohol fraction was diluted with an equal amount of water and chilled. Calcium phosphate (second gel) was added in amounts which would adsorb only a small per cent of the liver phosphorylase. The amount was determined by pilot experiments and was usually about 1.25 ml. of gel per 10 ml. of enzyme preparation. After 15 minutes of stirring, the gel was removed by centrifugation and discarded. The supernatant fluid still contained some carbohydrate as determined by the anthrone method (13), but on a protein basis possessed a high specific activity (Table II). The supernatant solution was diluted 10-fold with cold glass-distilled water; then the third addition of calcium phosphate gel was made in an amount sufficient to adsorb almost all the liver phosphorylase, 0.4 ml. per 10 ml. usually being sufficient. After 15 minutes of stirring, the gel was collected by centrifugation and washed with cold 0.001 M Tris (pH 7.4) with a volume equal to

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[4] Higher concentrations of citrate will make all the calcium phosphate gel soluble. The amounts of citrate used here, however, did not greatly influence the amount of insoluble calcium phosphate. The use of citrate for elution of calcium phosphate gel may be helpful when a protein is strongly adsorbed to the gel.

$\frac{2}{3}$ the discarded supernatant fluid. The enzyme was eluted from the gel by repeated additions of 10 ml. portions of cold 0.6 M ammonium sulfate (pH 7.0 to 7.2). Four such elutions were sometimes necessary. This adsorption-elution procedure did not increase the specific activity but did decrease the amount of carbohydrate considerably, as shown in Table II.

Step 9. Final Ammonium Sulfate Precipitation—The eluate of the third gel was added to 1.5 volumes of Versene-treated saturated ammonium sulfate solution which had been chilled previously. After 15 minutes at 3°, the precipitate was collected by centrifugation and dissolved in about 2 ml. of water. This final fraction was dialyzed *versus* water containing 7×10^{-4} M KOH, then *versus* 3×10^{-4} M KOH, and finally *versus* neutralized distilled water. Such purified preparations were unstable to freezing, un-

TABLE II

Summary of Purification and Yield; Step 5 through Step 9

Fraction	Total units	Specific activity	Per cent original activity	Carbohydrate / Protein
Step 5. 3rd alcohol	6000	8.8	100	20:1
" 6. Eluate of gel	5200	14.3	87	1.8:1
" 7.				
4th alcohol	4100	15.5	68	1.3:1
After dialysis and centrifugation	4100	22.4	68	1:1.1
Step 8.				
2nd gel supernatant fluid	3320	28.0	55	1.2:1
Eluate, 3rd gel	2930	24.6	48	1:8
Step 9. Final ammonium sulfate	2370	28.5	39.5	1:24

less protected by glycylglycine as described below. Further ammonium sulfate precipitation was possible and reduced the carbohydrate content to very low levels. At times the preparations of Step 8 or Step 9 were centrifuged for 40 minutes at $100,000 \times g$ at about 3° to remove traces of sedimentable material.

Properties of Liver Phosphorylase

Various preparations of purified liver phosphorylase possessed specific activities of 28 to 30. When activity was determined at 30° under conditions for which first order kinetics were followed, these preparations were found to possess an activity of about 3800 units per mg. of protein, as defined by Cori *et al.* (12). Liver phosphorylase with a specific activity of 18 was reported homogeneous when examined by ultracentrifugal and electrophoretic techniques and had a molecular weight calculated to be 237,000 gm. (7). Physical studies of the more active recent preparations (specific

activity of 2S) have been limited to a determination of the sedimentation constants. The value of s_{20} was found to be 8.4, which agrees with the previously determined sedimentation constants.[5]

Liver phosphorylase was very soluble in water or in the presence of various dilute salts. When freed of carbohydrate, the enzyme slowly lost activity at 3°, or at room temperature, and rapidly lost activity on freezing at −20°. The enzyme was relatively stable when frozen at −20° in glycylglycine buffer (0.2 M, pH 7.4), although repeated freezing and thawing in this buffer resulted in some loss of activity. The enzymatic activity was increased 15 to 40 per cent by the addition of 5-AMP or inosine-5-phosphate to the reaction mixture in final concentration of 10^{-3} M. Cysteine and glutathione did not stimulate purified liver phosphorylase, although the inhibition of liver phosphorylase by p-chloromercuribenzoate (10^{-4} to 10^{-5} M) was reversed by these compounds. The enzyme was inhibited by heavy metals; *e.g.*, strong inhibition was caused by addition of 10^{-6} M Hg^{++}, 10^{-5} M Ag^{+}, or 10^{-3} M Cu^{++} to the reaction mixture. Since the enzyme is sensitive to heavy metals, it seems likely that some preparations may be stimulated by metal-binding agents, but to date little or no stimulation by metal-binding agents has been observed.

The pH optimum for activity was near 6.4; the activity fell rapidly below pH 6.0 and above pH 7.0. Glycogen was a necessary component in the reaction mixture, and the purified enzyme catalyzed the formation of a polysaccharide which gave a blue color with iodine. During early fractionation liver phosphorylase accompanied the glycogen; moreover, when freed of glycogen, it still tended to associate with added glycogen. This could be shown by comparing sedimentation at $100,000 \times g$ without and with added glycogen; phosphorylase remained in the supernatant fluid after 40 minutes of centrifugation without glycogen, but in the presence of added glycogen was found in the sediment.

SUMMARY

The preparation of purified liver phosphorylase has been described. This procedure resulted in greater yield of the enzyme with higher specific activity than reported previously.

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THE RELATIONSHIP OF EPINEPHRINE AND GLUCAGON TO LIVER PHOSPHORYLASE

II. ENZYMATIC INACTIVATION OF LIVER PHOSPHORYLASE*

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The inactivation of liver phosphorylase in slices or extracts is catalyzed by an enzyme which was designated liver phosphorylase-inactivating enzyme (1). The inactivated phosphorylase formed in slices could be reactivated following addition of epinephrine or glucagon. In order to study the inactivation of purified LP[1] the inactivating enzyme was purified to a degree adequate for these studies. The soluble purified enzyme, on incubation with LP, caused a release of inorganic phosphate coincident with the loss of phosphorylase activity (2). The release was measured chemically in TCA supernatant solutions of enzymatically inactivated LP, and also by radioactivity when the substrate was radioactive phosphorylase (3). Ultracentrifugal analysis of LP and inactive LP showed that the two forms had the same sedimentation constant. The term inactive LP was used since activity was not restored when tested in the presence of 5-AMP, another example of differences existing between phosphorylase forms in liver and muscle. This paper deals with the purification, properties, and mode of action of the inactivating enzyme from liver.

Methods

Materials—The liver phosphorylase used routinely as a substrate in these studies was the third alcohol fraction (4) which had been dialyzed overnight against water. These preparations were stable for months at −20° and withstood repeated freezing and thawing. For the large scale inactivations, dialyzed samples of LP of Step 9 (4) were used. In some cases these were precipitated with 2 volumes of cold neutral Versene-treated ammonium sulfate previously saturated at room temperature. The precipitate was dissolved in cold water, centrifuged for 40 minutes at 100,000 × *g*, repre-

[1] The following abbreviations are used: LP, dog liver phosphorylase; IE, liver phosphorylase-inactivating enzyme; Tris, tris(hydroxymethyl)aminomethane; G-1-P, glucose-1-phosphate; 5-AMP, adenosine-5-phosphate; TCA, trichloroacetic acid.

cipitated as above, dissolved in a minimal amount of cold water, and dialyzed. The specific activity of these samples varied between 20 and 31.

Glycogen, G-1-P, Tris, ammonium sulfate, and calcium phosphate gel were prepared as described in Paper I (4). The ammonium sulfate solutions used were saturated at room temperature and neutralized with concentrated NH_4OH. Sodium sulfate was recrystallized from an aqueous solution saturated at 45° containing 0.6 gm. of disodium Versene per liter. After standing overnight at 0°, the crystals were washed successively with ethanol, ethanol and ether (1:1), and ether. The dried crystals were heated for about 24 hours at 110°.

Caffeine and 5-AMP were purchased from the Nutritional Biochemicals Corporation. Phosvitin was purchased from the Worthington Biochemical Corporation. The samples of α- and β-casein were generously supplied by Dr. T. L. McMeekin of the Eastern Regional Research Laboratory.

Analytical Methods—The analytical procedures used were as previously described (4), except that a number of analytical procedures were adapted to 0.5 ml. systems and absorptions were measured in Pyrocells in a Beckman model DU spectrophotometer. Among the procedures adapted were phosphorus determinations by the methods of Fiske and Subbarow (5) and Lowry and Lopez (6), pentose with the use of orcinol (7), carbohydrate with anthrone (8), glucose according to Nelson (9), and ashing of samples with perchloric acid as described by Bessey *et al.* (10).

Tissue Samples—Samples for the enzyme survey were obtained from a young male dog which had been anesthetized with ether and bled by severing the neck vessels. Samples of various tissues were placed in test-tubes, frozen immediately in a dry ice-alcohol bath, and then stored at −20° until ready for assay. In most cases the extracts for the survey were prepared by grinding samples of the tissues in a cold TenBroeck glass homogenizer with 10 to 20 volumes of cold water. The protein concentration of the various homogenates for the survey was determined by the method of Weichselbaum (11).

Standard Assay—The activity of IE was determined by measuring the amount of LP inactivated during a 10 minute incubation with IE at 37°. The reactions were started by the addition of 0.2 ml. of IE (about 1 γ of purified enzyme) to a mixture at room temperature containing 0.5 ml. of 0.1 M Tris (pH 7.2), 0.2 ml. of caffeine (1.0 mg. per ml.), approximately 0.75 unit of LP, and water to a final volume of 1.5 ml. After a 10 minute incubation at 37°, 0.5 ml. of a solution containing 5 per cent G-1-P, 1.5 per cent glycogen, and 0.3 M NaF (adjusted to pH 6.1 with HCl) was added and the residual LP was determined by measuring the rate of liberation of phosphate from G-1-P. After an additional 10 minutes at 37°, a 0.5 ml. aliquot of the reaction mixture was transferred to cold TCA and the amount of

phosphate present in an equivalent of 0.1 ml. of reaction mixture was determined. The per cent of LP inactivated was determined by the difference from a corresponding control in which IE was omitted; values were used when the extent of inactivation was less than 65 per cent. The rate of inactivation with purified IE was almost linear to 50 per cent inactivation; however, correction factors were used in the 50 to 65 per cent range in order to convert to linearity; for example, the factor at 55 per cent was 1.05, at 60 per cent 1.10, and at 65 per cent 1.15. 1 unit of inactivating enzyme was defined as that amount which catalyzed the inactivation of 1 unit of LP in the above assay. The specific activity was expressed as units per mg. of protein.

Results

Purification Procedure

Steps 1 and 2—The first two steps of purification were the same as those used in purifying LP (4). The animal was sacrificed, the liver was perfused with fluoride, and, after homogenization in a Waring blendor, a filtrate was obtained by use of a Hormann filter press. The specific activity of the homogenate varied between 1.1 and 1.6, while that of the filtrate was about 2. The inactivating enzyme was adsorbed on calcium phosphate gel as described and the supernatant solution was used as a source of phosphorylase. These and all subsequent steps were carried out at 0–3° unless specified otherwise.

Step 3. Gel Washes and Elution—The gel was washed twice with 0.02 M Tris (pH 8.0) containing 10^{-3} M $MgCl_2$ (1 liter per 300 to 400 gm. of liver), then washed three times in a similar fashion except that the buffer was at pH 9.0. The last wash was 0.5 the volume of the previous washes in order to collect the gel in one centrifuge bottle. Glass-distilled water was used routinely, and gel collections were made by 10 minute centrifugations at 500 $\times$ g.

Elution was carried out at 30° for 10 minutes, a volume of a solution of 0.05 M Tris (pH 10) and 0.3 M Na_2SO_4 equal to one-half the weight of the tissue being employed. The eluted gel was collected by centrifugation at 7000 $\times$ g at room temperature for 10 minutes and discarded. The clear yellow eluate was chilled in an ice water bath and adjusted to pH 8.0, as measured with the glass electrode, with 1 N acetic acid. Adsorption of the enzyme on the gel, combined with extensive washing and subsequent elution, resulted consistently in a 40-fold or greater purification. While purification at this step was consistently good, the yields were variable.

Step 4. First Ammonium Sulfate Fractionation—Ammonium sulfate precipitates were collected after waiting for 10 minutes by centrifugation

for 10 minutes at approximately 7000 × g. $\frac{2}{3}$ volume of saturated ammonium sulfate was added to the eluate. The enzyme was precipitated from the supernatant solution by adding 23 gm. of ammonium sulfate and 1 ml. of 1 N KOH per 100 ml. of eluate. The precipitate was dissolved in 0.1 M Tris (pH 10) by using 3.0 ml. per 100 gm. of tissue. This step resulted in almost 2-fold purification.

Step 5. Second Ammonium Sulfate Fractionation—A fraction was removed by the addition of 6/11 volume of saturated, Versene-treated ammonium sulfate. This step was somewhat variable and often an additional drop or 2 of ammonium sulfate were required to get the slight desired haze. The solution was centrifuged and the precipitate was discarded. The supernatant solution was made more alkaline by the addition of 0.5 N KOH (2 ml. per 17 ml. of solution) and was brought to 50 per cent saturation with saturated ammonium sulfate. The precipitate was collected and dissolved in 1 ml. of 0.2 M Tris (pH 9.0) for testing. The enzyme was quite stable when lyophilized and lyophilization was carried out after the addition of 2 ml. of 0.2 M Tris (pH 9.0) per ml. of enzyme solution. Specific activities of this fraction ranged between 200 and 300 with about 10 per cent recovery of the total activity in the homogenate.

Step 6. Ethanol Fractionation of Lyophilisate—The powder was stable for weeks when stored in the cold under vacuum and portions could be purified further when needed. For example, 18 mg. of powder were dissolved in 1.5 ml. of 0.05 M Tris (pH 8.0) and 1.5 ml. of 1 per cent NaCl. The enzyme was precipitated by adding 1.5 ml. of 80 per cent ethanol (−20°). The solution was allowed to stand for 7 minutes in an ice water bath and was centrifuged for 7 minutes at approximately 1000 × g at 0–3°. The precipitate was dissolved in 1 ml. of 1 per cent NaCl. Such preparations were generally used, but often these samples were heated for 15 minutes at 50° before use. Fractionation with ethanol often resulted in a 2-fold purification with 50 to 70 per cent recovery of activity. These preparations were soluble and the activity remained in the supernatant solution when centrifuged for 40 minutes at about 75,000 × g.

Distribution of Enzymes—The results of a survey of various dog tissues are shown in Table I. The analyses were carried out under standard conditions with and without the presence of 0.1 M NaF during the incubation of LP with the tissue sample. Inhibition by fluoride was taken to be indicative of the presence of an inactivating enzyme (or enzymes) similar to the one present in liver. Pancreatic extracts, for example, resulted in complete inactivation of LP even in the presence of fluoride, presumably due to the action of proteolytic enzymes. In blood an inactivating enzyme was found to be present in the red blood cells while little or no inactivating enzyme was found in the plasma.

Properties of IE

Stability of Enzyme—The condition found most suitable for storage of the purified enzyme was the lyophilized state and little activity was lost over a period of weeks at $0°$ *in vacuo*. The enzyme, after fractionation with alcohol, usually stored well at $3°$ for a day or 2 in relatively high concentrations of NaCl and could be incubated at $50°$ for 15 minutes to inactivate contaminating enzymes, such as small amounts of pyrophosphatase, with little or no loss of IE activity. The enzyme was not stable in a dilute state for extended periods and all activity determinations were

TABLE I

Distribution of Inactivating Enzymes in Dog Tissues

Tissue	Specific activity*
Liver	1.2–1.6
Kidney	1.2
Spleen	0.5
Pituitary	0.4
Base of brain	0.1
Brain cortex	0.1
Lung	1.0
Skeletal muscle	0.3
Heart	0.3
Bladder	0.8
Intestine	0.4
Intestinal mucosa	0.6
Femoral artery	0.4

* See the text for unit of measurement.

made just after dilution in 0.005 M Tris (pH 7.2). Enzymatic activity was lost rapidly below pH 5.5.

pH Optimum—The effect of pH on the enzymatic inactivation of LP was determined by substituting 0.01 M buffers for 0.1 M Tris (pH 7.2) in the standard assay. This lower buffer concentration was chosen so as to avoid pH changes in the final analysis of phosphorylase activity. The optimum for activity was near pH 8, as shown in Fig. 1; stability measurements accompanying the measurement of the pH optimum showed that this was not a result of an increased stability of the enzyme. Glycylglycine and Tris buffers served equally well, Veronal and glycine were less satisfactory, while citrate and pyrophosphate were unsuitable since they inhibited 70 per cent or more at 10^{-2} and 10^{-3} M, respectively.

Effect of Substrate Concentration—Since the substrate molarity was very low (7×10^{-8} M), it was of interest to determine whether IE was saturated

at this low level. Standard assays were carried out, except for varying the initial LP concentrations and making appropriate dilutions of the reaction mixtures to carry out assays for the residual phosphorylase. It was found that IE was not saturated with substrate in the amounts used in the standard assay or by 10 times that amount of LP.

Effect of Various Compounds on Rate of IE Activity—The effect of various ions on IE activity was studied by separate additions to the standard assay. At the same time the effect on controls containing LP
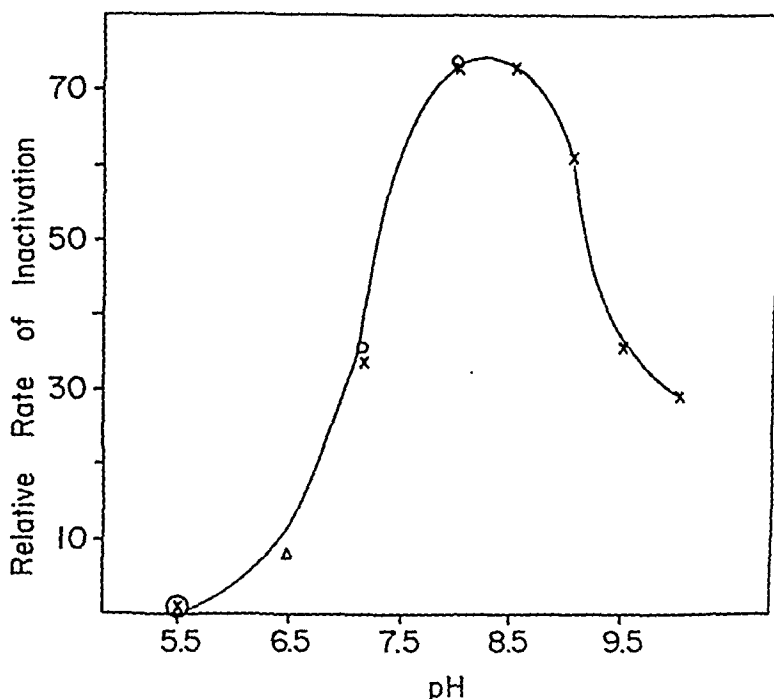
Fig. 1. Effect of pH on IE activity. The standard assay was used with 0.01 M buffer as indicated in place of 0.1 M Tris. ⊗ = acetate, × = Tris, ○ = glycylglycine, △ = imidazole.

and no IE was studied. At final concentrations of 5×10^{-4} M, Na$^+$, K$^+$, and Mg^{++} had no influence on the rate of the enzymatic reaction, while at final concentrations of 5×10^{-5} M and lower, Hg^{++}, Al^{+++}, and Zn^{++} markedly decreased IE activity. A number of salts used in purification exerted inhibitory effects at concentrations of 0.013 M and greater, *e.g.* NaCl, Na$_2$SO$_4$, and (NH$_4$)$_2$SO$_4$. NaF was found to be an inhibitor of IE and 90 to 100 per cent inhibition was approached when the final concentration was 0.01 M. A number of other substances tested for their effects on the reaction included Versene, glutathione, and cysteine which appeared to have no significant effect at 10^{-3} M concentration.

The purified enzyme was inhibited by 5-AMP and inosine-5-phosphate, as noted previously when crude enzyme was studied (1). The activity of

the purified enzyme on liver phosphorylase was stimulated by the addition of caffeine and theobromine, as reported previously (1), and also by menadione.

Products of Enzymatic Inactivation of LP

Formation of Inorganic Phosphate—With purified preparations of inactivating enzyme and liver phosphorylase, experiments were performed in order to determine whether some small group might be formed during the inactivation reaction. Since the substrate in these reactions was of high molecular weight, it was necessary to use relatively large amounts of the dialyzed enzyme (200 to 500 units of phosphorylase purified through Step 9 (4) or further). Phosphorylase was incubated at 25° for 30 to 60 minutes with and without the addition of IE protein usually in amounts varying between 0.5 and 2.5 per cent of the amount of LP protein. The pH of the reaction was determined by that of the two protein solutions used and was usually about 7.3. Following incubation, some inactivated samples were dialyzed *versus* small volumes of water and the dialysates were compared with those from controls. In most cases inactivated samples were treated with TCA or perchloric acid and, after centrifugation, the supernatant solutions were compared with those from controls. A comparison of dialysates or TCA supernatant fluids of phosphorylase with and without enzymatic inactivation revealed no differences in amounts of pentose, ultraviolet-absorbing material, phenolic type compound, or peptide. Inorganic phosphate, however, was formed from LP during inactivation and, as reported earlier, the time-course of liberation of phosphate from LP was coincident with the loss of LP activity (2). Table II summarizes an experiment in which the amount of phosphate released paralleled the loss of enzymatic activity. It can be seen that negligible amounts of TCA-soluble phosphate are released from LP or IE alone and that release of phosphate from the two together ceases when inactivation ceases. The amount of phosphate liberated was essentially the same when measurements were made for stable phosphate or for phosphate as determined by the method of Fiske and Subbarow or by the procedure of Lowry and Lopez. From ten experiments the amount of phosphate liberated was calculated to be 0.3 γ of P per mg. of LP (on the basis that 30 units of LP equal 1 mg. of LP) or about 1 mole of phosphate per 100,000 gm. of enzyme. This appears to represent about 25 per cent of the total phosphate present in these preparations as determined by ashing (10). Whether this extra phosphate is related to LP or to a contaminant is not yet clear, but treatment with Norit or incubation with ribonuclease does not appreciably lower the phosphate content, nor is the extra phosphate released on prolonged incubation with IE as shown in Table II.

The formation of TCA-soluble phosphate during inactivation was also measured by the use of isotopes. Purified radioactive (P^{32}) phosphorylase was prepared for use as a substrate, as described in Paper III (12), by incubating liver slices with P^{32}-orthophosphate and epinephrine. Table III summarizes an experiment in which chemical measurements of TCA-soluble phosphate were accompanied by measurements of isotopic phosphate in both TCA supernatant fluids and precipitates. It can be seen that the radioactive phosphate (which had been incorporated into LP in slices) remained with the TCA precipitate before enzyme inactivation, but that most of the radioisotope became TCA-soluble during enzymatic

TABLE II

Inorganic Phosphate Formation during Enzymatic Inactivation of LP

To 15.5 mg. of LP in 1.3 ml. was added 0.05 ml. of IE (20 γ of protein) and the mixture was incubated at 25°, as were the samples of IE and LP alone. At the indicated times small aliquots were removed for activity determinations in the standard LP assay containing 5-AMP. Release of inorganic phosphate from LP was measured after sampling 0.2 ml. aliquots into 0.4 ml. of 7.5 per cent TCA. Following centrifugation, 0.4 ml. of the supernatant solution was used in the analysis.

Time	IE	LP		IE + LP	
	γ P*	γ P per mg. LP	Per cent activity	γ P per mg. LP	Per cent activity
min.					
0	0.03	0.01	100	0.02	100
50				0.33	36
95	0.01	0.02	105	0.32	27
300			94	0.32	25

* In this case the amount of P found in the TCA supernatant fluid was not related to the mg. of LP but to a volume equivalent to that containing 1 mg. of LP.

inactivation. These results and others (12) indicate that only the phosphate which is released from LP by IE equilibrates rapidly with phosphate within the slice.

Specificity Studies—The liberation of phosphate from LP prompted a number of experiments to determine whether or not the purified heat-treated inactivating enzyme catalyzed the release of phosphate from other phosphoproteins and non-protein phosphate esters. Non-protein phosphate esters at a final concentration of 10^{-3} M were incubated in the usual test system, except that caffeine was omitted, since it was not a necessary component for the release of phosphate from LP. 100 to 200 times the usual amount of IE was used and incubations were carried out for as long as 4 hours at 25°. In each case the effect of Mg^{++} (10^{-3} M) was tested. After incubation, 0.5 ml. aliquots were added to 0.8 ml.

portions of 10 per cent TCA. Following centrifugation, 1.0 ml. aliquots were analyzed for phosphate by the method of Fiske and Subbarow, adapted to a 1 ml. system. IE was found to liberate no significant amounts of phosphate from α-glycerophosphate, p-nitrophenyl phosphate, 5-AMP, adenosine diphosphate, adenosine triphosphate, creatine phosphate, phosphoenolpyruvate, glucose-1-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate, or pyrophosphate even in the presence of Mg^{++}.

Several known phosphoproteins were incubated with IE under conditions similar to those described above. Phosphate was found to be released from phosvitin, and on longer incubation approximately 10 per

TABLE III

Formation of TCA-Soluble Phosphate Measured by Isotopic and Chemical Methods

To 9.5 mg. of LP in 1.9 ml., having a total of 895 c.p.m. (94 c.p.m. per mg. of LP), was added 0.05 ml. of IE (46 γ of protein). Incubation was carried out at 25° and 0.5 ml. aliquots were transferred to TCA at the indicated times. After measuring P chemically in the supernatant solution, aliquots of the phosphomolybdate complex were dried and counted. The TCA precipitates were transferred to planchets, dried, and counted

Incubation time with IE	TCA fraction	C p m per mg LP	P per mg LP
min			*γ*
0	Supernatant	5	0.13
	Ppt.	82	
85	Supernatant	75	0.45
	Ppt.	26*	
Δ			0.32

* Since the precipitates were not washed, an appreciable portion of supernatant solution was present in the precipitate; after correction this value would be lower.

cent of the total phosphate appeared as inorganic phosphate. Smaller amounts of inorganic phosphate were released when α-casein was the substrate, while no measurable amounts of phosphate were released from β-casein or ovalbumin. A comparison was made of the action of IE on LP and phosvitin and, as shown in Table IV, the action of the enzyme on both substrates was inhibited by NaF and NaCl. Enzyme activity was not stimulated by Mg^{++}, and the enzymatic activities were parallel when heat-treated as indicated.

No proteolytic activity was found when purified IE was tested with several proteins as possible substrates.

Characteristics of Inactive LP—The effect of enzymatic inactivation on the sedimentation constant was determined by ultracentrifugal analysis of LP with and without enzymatic inactivation. Prior to inactivation the

TABLE IV

Comparison of IE Activity on LP and Phosvitin

The activity towards LP was studied in the standard IE assay with the indicated modifications. The heated samples of IE were lyophilized samples put through an alcohol step, adjusted with acetate buffer or Tris, heated as indicated, then chilled, neutralized, and tested. The activity towards phosvitin was studied by use of the modified assay in which caffeine was omitted and LP was replaced by 1.8 mg. of dialyzed phosvitin. The reactions were stopped with TCA after 3 hours incubation at 25° and phosphate was measured in the supernatant fluid.

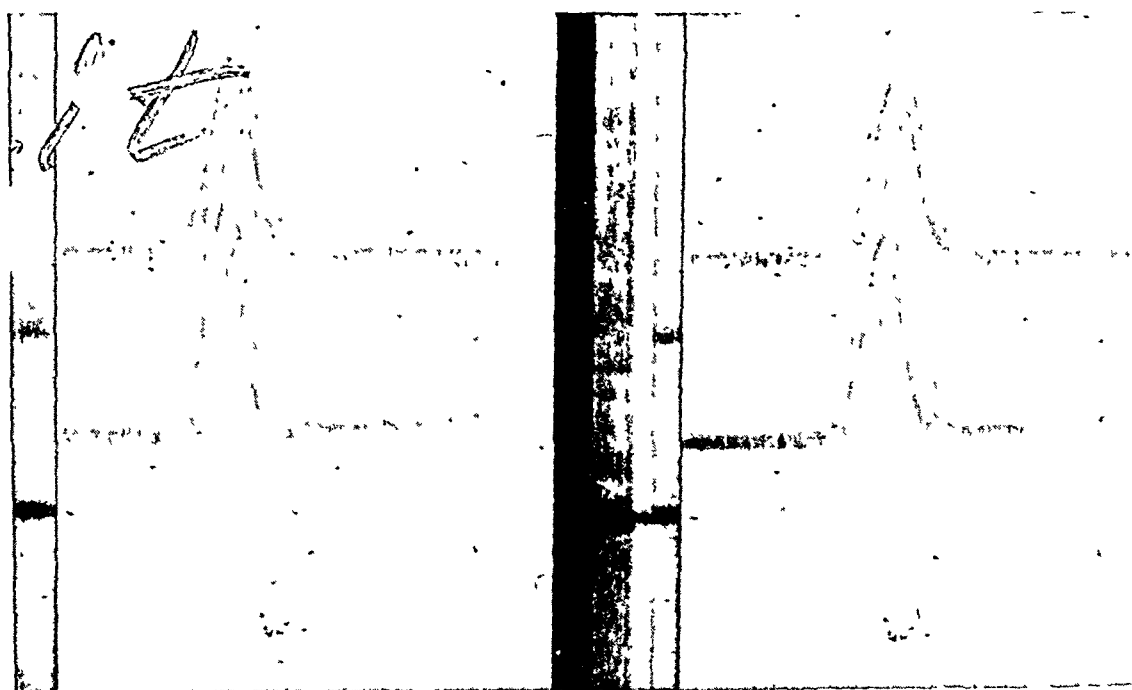| Treatment | Effect on activity towards | |
	LP	Phosvitin
0.15 M NaCl in reaction mixture	78% inhibition	78% inhibition
0.01 " NaF " " "	95% "	70% "
0.002 M MgCl₂ in reaction mixture	No effect	No effect
Pretreated for 15 min. at pH 4.0 and 37°	Complete inactivation	Complete inactivation
Pretreated for 15 min. at pH 8.0 and 50°	60-85% recovery	60-85% recovery

FIG. 2. Ultracentrifugation of LP and inactive LP. Typical sedimentation patterns of active LP (upper pattern) and inactive LP (lower pattern) made simultaneously at 196,900 × *g*. Two time periods, showing sedimentation at 32 and 40 minutes, are presented to illustrate the character of the peaks observed during sedimentation.

samples were dialyzed overnight *versus* 0.1 M NaCl and 0.002 M Tris, pH 7.4. To 16 mg. of LP in 2.0 ml. of the indicated media was added 0.46 mg. of IE in 0.2 ml. of the same media. The reaction mixture along with the corresponding control (containing all components except IE) was incubated for 1 hour at 25°. During this period the control lost no activity, while in the sample containing IE there was little or no LP activity. Both samples were redialyzed with continual shaking for 2 hours before ultracentrifugation. The samples were centrifuged in a Spinco model D ultracentrifuge at 196,900 $\times$ *g* and at 26.4°.[2] As indicated in Fig. 2, there was a single symmetrical peak for LP and inactive LP and both had the same sedimentation constant (s_{20} = 8.4) at identical enzyme concentrations.[2] The displacement of the peaks is a result of different bubble sizes in the two cells. The activities of the suspended samples after centrifugation were essentially the same as before centrifugation. Assay of the reaction mixture in the absence of 5-AMP indicated that 98 per cent inactivation had occurred, while assay in the presence of 5-AMP indicated that 78 per cent inactivation had occurred. As mentioned previously, inactive LP differs from a "*b*" form of phosphorylase in that enzymatic activity is not restored on addition of 5-AMP to the reaction mixture. Usually when complete inactivation occurred, as judged by assay in the absence of 5-AMP, approximately 85 per cent inactivation occurred, as estimated by assay in the presence of 5-AMP.

DISCUSSION

The study of an enzyme exerting its catalytic action on a substrate *in vitro*, which is also an intracellular enzyme from the same tissue, posed some special problems. In addition to the problem of obtaining substrate quantities of purified enzyme, the preparation of the substrate (LP) was complicated by the very presence of the catalyst (IE). Moreover, since the substrate was of high molecular weight, it would be expected that the yield of any small fragment would be low in relation to the total amount of substrate used. Experiments reported in this paper showed that the amount of small product formed (inorganic phosphate) was approximately 0.03 per cent of the substrate by weight.

In the standard IE assay the LP concentration was estimated to be 7×10^{-8} M. In liver cells, however, the concentration of phosphorylase is much higher and was estimated to be 1.8×10^{-6} M (a concentration approximately one-tenth that used in the large scale inactivations). The molar concentration of IE present in liver cells cannot yet be estimated,

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[2] Measurements performed at the Cleveland Clinic. M. D. Schoenberg, personal communication.

but the enzyme concentration in liver is sufficient to catalyze the inactivation of all LP present in several minutes or less.

All evidence to date indicates that IE is a phosphatase. No materials except phosphate and inactive LP were found to be products of the inactivation reaction; hence liver phosphorylase phosphatase might be considered an appropriate name for the enzyme. Evidence supporting this view is reported in Paper III (12) where it is shown that inactive LP, after extensive dialysis, could be reactivated in the presence of another enzyme fraction and adenosine triphosphate. Phosphatase activity was not exhibited when IE was incubated with a number of simple organic phosphate esters, but some phosphate was released from phosvitin and α-casein. The amounts of phosphate released were small, however, and it is not entirely clear that phosphoserine proteins are the necessary substrates for IE. The nature of the organic phosphate moiety in LP is currently under investigation and future results may permit a more exact descriptive name for the enzyme.

The dephosphophosphorylase (inactive LP) formed during the reaction sedimented at the same rate as the active form. Thus, there did not appear to be a gross change in the phosphorylase molecule, as was reported when phosphorylase a from rabbit muscle was incubated with the phosphorylase-rupturing enzyme (13). Therefore, the exact relationship of IE to the phosphorylase-rupturing enzyme is not clear, especially since the enzymes studied were different in origin and properties.

Results to be published at a later date indicate that IE from dog heart muscle is similar to the one from dog liver. Highly purified inactivating enzyme from dog heart was also found to catalyze the release of inorganic phosphate from liver phosphorylase as did the IE from dog liver.

SUMMARY

1. The purification and properties of a soluble liver phosphorylase-inactivating enzyme from dog liver have been described.

2. The inactivating enzyme catalyzed the conversion of liver phosphorylase to an inactive form whose activity was not restored by adenosine-5-phosphate. Products of the enzymatic inactivation were inactive liver phosphorylase and inorganic phosphate. Inactive liver phosphorylase sedimented at the same rate as the active form.

3. The inactivating enzyme exhibited phosphatase action when incubated with liver phosphorylase and slight phosphatase activity was noted on incubation with phosvitin and α-casein. No phosphatase activity was noted, however, on incubation with a number of simple organic phosphate esters.

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THE RELATIONSHIP OF EPINEPHRINE AND GLUCAGON TO LIVER PHOSPHORYLASE

III. REACTIVATION OF LIVER PHOSPHORYLASE IN SLICES AND IN EXTRACTS*

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Evidence has been presented which showed that the enzymatic inactivation of liver phosphorylase was accompanied by the removal of phosphate from the enzyme (1, 2). It was reasoned that the conversion of dephosphophosphorylase (inactive LP)[1] to the active enzyme should involve the addition of phosphate to the molecule. Therefore, the reactivation process was studied in liver slices with P^{32}-orthophosphate to detect entrance of phosphate into the enzyme and also in liver extracts with purified dephospho-LP as substrate.

It was found that P^{32}-orthophosphate was rapidly incorporated into phosphorylase in liver slices and this incorporation was greatly increased under the influence of epinephrine and glucagon. Furthermore, a soluble liver enzyme[2] capable of converting dephospho-LP to LP in the presence of ATP and magnesium ions was found and purified.

Methods

Preparation of Liver Slices—Healthy adult dogs under secobarbital anesthesia were sacrificed and bled by severing the neck arteries. After cannulation of the portal vein, the livers were perfused with cold 0.9 per cent NaCl, then removed and chilled for 10 minutes in 0.9 per cent NaCl.

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[1] The following abbreviations are used: LP, liver phosphorylase; dephospho-LP, liver dephosphophosphorylase; IE, liver phosphorylase-inactivating enzyme; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid.

[2] This enzyme has been named liver dephosphophosphorylase kinase thus conforming to current terminology. For convenience phosphokinase is used as an abbreviation. It is pointed out that the use of the term kinase also conforms to an early definition in which a kinase was a substance which transformed a zymogen to an enzyme.

Slices, measuring on the average 25 mm. × 20 mm. × 2 mm., were cut free-hand in the cold room. 15 gm. portions of the pooled slices were transferred to 250 ml. Erlenmeyer flasks and washed at least once by swirling for 1 minute in 5 to 6 volumes of cold 0.9 per cent NaCl. After decantation of the wash fluid through gauze, 2 volumes of incubation medium (pH 7.4, 37°) were added to each vessel, which was then stoppered with a 2-hole rubber stopper and shaken in a bath at 37° at 120 oscillations per minute. The experiment was terminated by homogenizing the slices in a Waring blendor at 2°. The homogenates contained NaF at a final concentration of 0.1 M.

Modification of LP Preparation—For some purposes the standard LP preparation procedure (3) was modified. The standard procedure was followed to the collection of the fourth alcohol fraction (Step 7). The precipitate was suspended in 0.1 M phosphate buffer (pH 7.2) and incubated at 25° for 30 to 40 minutes. The resulting precipitate was removed by centrifugation and the supernatant fluid (containing LP) was dialyzed against alkalinized water (2 × 10^{-5} N KOH) for 20 hours at 2°.

Purification of Radioactive Phosphorylase—The homogenates of the liver slices were centrifuged for 20 minutes at 7000 × g. Radioactive phosphorylase was purified from the supernatant solution either by following the standard procedure from Step 2 through Step 9, omitting Step 8, or by the modified procedure described above. Carrier dog liver phosphorylase was added at various steps during the purification of the radioactive enzyme.

Preparation of Dephospho-LP—In order to study the reactivation process in extracts, it was desirable to prepare batches of dephospho-LP which could be stored and used periodically. The stability of a dephospho-LP preparation to storage under various conditions appeared to parallel the stability of the LP preparation prior to enzymatic inactivation. For this reason preparations containing enough endogenous glycogen to stabilize them to freezing and thawing were used. For routine preparation of dephospho-LP about 45 mg. of LP (prepared by the modified procedure above) were incubated at 27° with about 150 units of IE (an alcohol fraction heated at 50° for 15 minutes (2)). Inactivation was allowed to proceed to completion, as judged by assay of activity in the absence of adenosine-5-phosphate (usually 60 minutes were required). The preparation was then dialyzed *versus* alkalinized water for several hours in the cold with agitation and was stored at −20° in fifteen to twenty portions. Preparations of dephospho-LP obtained from more highly purified LP, or dialyzed more extensively (up to 24 hours), were reactivated at the same rate and to the same extent as those prepared as described above.

Assay Procedures—Assays of phosphorylase and microdeterminations of

inorganic phosphate were made as described previously (2, 3). Assays of phosphokinase were carried out in a system containing (at final concentrations) 1×10^{-3} M ATP, 2.5×10^{-3} M $MgSO_4$, 2.0×10^{-2} M NaF, 1×10^{-2} M Tris buffer (pH 7.4), 0.2 mg. per ml. of dephospho-LP, and the enzyme solution to be assayed. Aliquots of the reaction mixture were removed at zero time and after 10 minutes incubation at $37°$ and were assayed for LP activity in the standard system (3). Radioactivity measurements were made on dry samples with an end window Geiger-Müller tube.

Materials—Materials used in enzyme preparations and assay systems were prepared as described previously (3). P^{32}-Orthophosphate, obtained from the Oak Ridge National Laboratory, was diluted with 0.02 M glycylglycine buffer (pH 7.4) before use. Epinephrine was obtained commercially as a 1 per cent solution; glucagon was kindly donated by Eli Lilly and Company. Adenosine triphosphate, inosine triphosphate, uridine triphosphate, cytidine triphosphate, and adenosine diphosphate were obtained as the disodium salts from various commercial sources.

Results

Conversion of Dephosphophosphorylase to Phosphorylase in Liver Slices

Effect of Epinephrine and Glucagon on Incorporation of P^{32} into Phosphorylase—The response of dog liver slices to epinephrine or glucagon was similar to that observed when rabbit liver slices were used (4). Fig. 1 shows the effect of incubating dog liver slices for varying periods of time at $37°$ on the concentration of phosphorylase. The concentration decreased rapidly, reaching a minimal value after 15 to 20 minutes. Subsequent addition of epinephrine (or glucagon) resulted in an extremely rapid rise in the phosphorylase level (within 5 minutes). It should be pointed out that, unlike rabbit liver, dog liver phosphorylase decayed appreciably during the slicing procedure; the zero time concentration (Fig. 1) was lower than that in the liver analyzed before slicing.

With the time relationships of Fig. 1 as a guide, dog liver slices were incubated in the presence of P^{32}-orthophosphate with and without the addition of epinephrine or glucagon. The results of an experiment of this type are summarized in Table I. Three 15 gm. portions of liver slices were preincubated at $37°$ for 15 minutes in 30 ml. of 0.15 M glycylglycine + 0.001 M phosphate buffer containing 1 mc. of P^{32}. This preincubation period permitted the inactivation of phosphorylase and the entry of P^{32} into the slices. To one portion of slices 0.045 ml. of 1 per cent epinephrine was added (Sample E), to a second portion 0.66 mg. of glucagon was added (Sample G), and the third portion served as a control (Sample C). Incubation was continued for 5 minutes, at which time the incubation

media were decanted through glass tubes in the rubber stoppers. The slices were washed by swirling for 1 minute in 0.15 M KCl at 20°, the wash
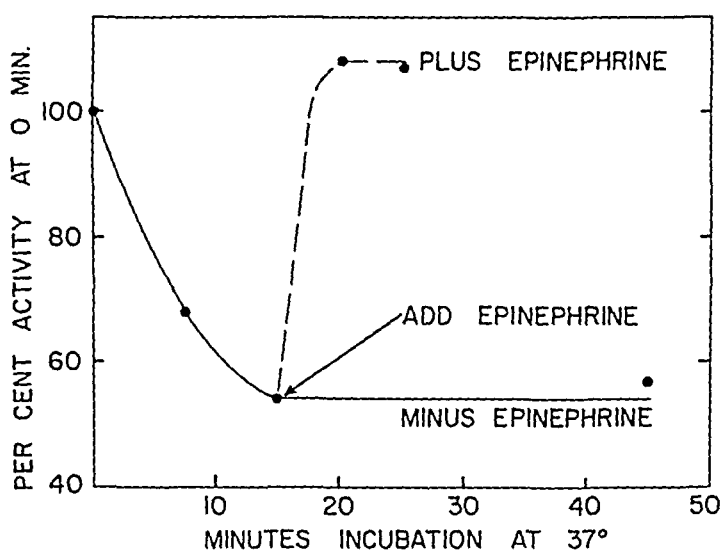
Fig. 1. Effect of epinephrine on phosphorylase concentration in dog liver slices. Seven 15 gm. portions of slices were incubated in 30 ml. portions of medium containing 0.15 M glycylglycine + 0.001 M phosphate buffer (pH 7.4). The slices were homogenized at the indicated times and LP activity was determined.

TABLE I

Effect of Epinephrine and Glucagon on Incorporation of P^{32} into Phosphorylase

15 gm. portions of liver slices were incubated for 20 minutes at 37° in 30 ml. of 0.15 M glycylglycine + 0.001 M phosphate buffer (pH 7.4). 1 mc. of P^{32} was added to each sample at zero time. 0.045 ml. of 1 per cent epinephrine or 0.66 mg. of glucagon was added after 15 minutes incubation. Phosphorylase was purified with carrier additions and enzymatically inactivated as described in the text.

	Control	Epinephrine	Glucagon
Units LP activity per gm. slice (before carrier addition)	5.1	9 9	8.1
Units LP activity per mg. protein in final preparation	27	29	29
% slice LP activity recovered	6 2	6 5	5.1
C.p.m. per % LP recovered	42.5	254	194
" made TCA-soluble after inactivation per % LP recovered	17.3	179	121

fluid being decanted as before, and homogenized in 40 ml. of cold distilled water containing added carrier phosphorylase. 7 ml. of 0.75 M NaF were added to each of the samples which were then homogenized for an additional minute. Each blendor was rinsed with 0.1 M NaF and the

rinse fluid was combined with the homogenate. By virtue of the carrier additions, each homogenate had approximately the same amount of phosphorylase activity. The phosphorylase was purified to a final specific activity of 27 to 29. As can be seen in Table I, the enzyme preparations derived from slices incubated with glucagon and epinephrine contained 4.5 to 6 times the radioactivity of the control preparation. Upon incubation of the final preparations with IE, most of the radioactivity became TCA-soluble. This point is illustrated in Fig. 2. Before exposure to IE, very little radioactivity was TCA-soluble, whereas, after complete
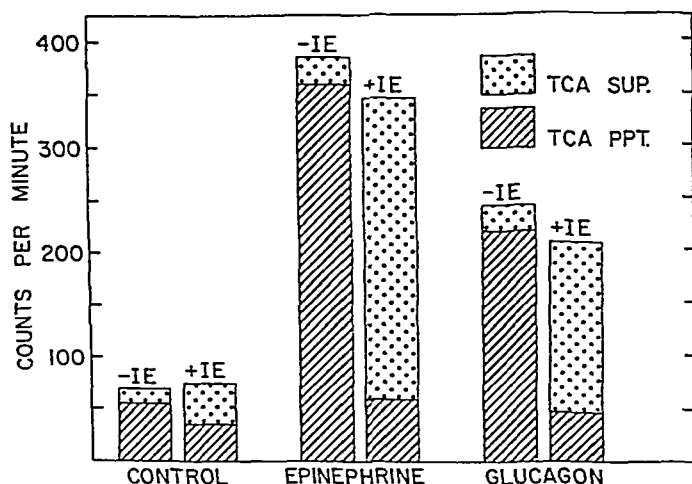
FIG. 2. Distribution of radioactivity in samples of radioactive LP precipitated by TCA. The LP samples were incubated for 30 minutes at 25° without and with IE and placed in 5 per cent TCA. The total height of each bar represents the sum of the radioactivity found in the unwashed precipitate and supernatant fluid after centrifugation.

inactivation, most of the radioactivity appeared in a TCA-soluble form. The difference between the TCA-soluble counts before and after inactivation is recorded in the bottom line of Table I. It can be seen that enzymatic inactivation of the radioactive phosphorylase derived from the glucagon- and epinephrine-treated slices resulted in the release of 7 to 10 times the amount of radioactivity released from the preparation derived from the control slices.

Non-Exchange of Purified Labeled Phosphorylase with P^{31}-Orthophosphate —Before the experiment discussed in the previous section was performed, a preliminary experiment had indicated that exchange of P^{32} in the radioactive enzyme with P^{31}-orthophosphate did not occur, even in the presence of glycogen. In this experiment aliquots of a sample of radioactive phos-

phorylase prepared from dog liver slices were incubated at 24° for 40 minutes. One aliquot contained phosphate buffer (0.01 M, pH 7.0), a second contained phosphate and glycogen (0.3 per cent), and a third contained only enzyme and water. After incubation all three aliquots had the same proportion of TCA-soluble and TCA-insoluble radioactivity (17 per cent soluble and 83 per cent insoluble).

In the experiment summarized in Table I it should be pointed out that carrier enzyme was added during homogenization of the slices and was thereafter exposed to P^{32} and the endogenous glycogen of the homogenate for some 60 minutes (at a temperature of 2–8°) before $(NH_4)_2SO_4$ was added. Furthermore, during the purification procedure, the labeled enzyme preparations were subjected to a 15 hour dialysis *versus* 0.1 M phosphate buffer (pH 7.2) at 21°. During this step, there is digestion of

TABLE II

Non-Exchange of Labeled Phosphorylase

Labeled enzyme preparations, derived from the experiment described in Table I and purified to an average specific activity of 24, were dialyzed for 15 hours at 21° *versus* 0.1 M phosphate buffer (pH 7.2).

Sample	C p.m. per mg. protein	
	Before dialysis	After dialysis
C	49.0	42.5
E	221	217
G	110	128

the glycogen contained in the preparation with resultant lowering of the carbohydrate content (3). As shown in Table II, under these conditions there was no loss of radioactivity during the dialysis, as would be expected if the P^{32} incorporated into the enzyme were to exchange with P^{31}-orthophosphate (or glucose-1-phosphate).

Effects of NaF, Epinephrine, and Glucagon on P^{32} Incorporation into Phosphorylase—Addition of NaF to preincubated rabbit liver slices also results in an increase in phosphorylase concentration (5). NaF is a known inhibitor of IE (2, 6) and presumably acts in slices by inhibiting the inactivation of phosphorylase. Since epinephrine and glucagon did not inhibit IE in extracts, it seemed possible that they acted by a different mechanism. However, no effect of these agents had been established in extracts, and it was not clear that a difference in mechanism of action existed (5). Therefore, the incorporation of radioactive phosphate into phosphorylase during incubation with epinephrine or glucagon was compared to incorporation in the presence of NaF.

Table III summarizes the results of an experiment in which liver slices were incubated in a NaCl-buffer medium containing epinephrine or glucagon or in a NaF-buffer medium. After 10 minutes incubation, 1 mc. of P^{32} was added and the incubation was continued for 5 minutes. The slices and media were homogenized in a solution containing appropriate amounts of NaF so that the final concentration in the homogenate was 0.1 M. The radioactive phosphorylase was then purified to a final specific activity of 9 to 13 by the modified purification procedure described under "Methods." In the last line of Table III is recorded the amount of radioactivity made TCA-soluble after enzymatic inactivation of the final phosphorylase preparations. In view of the data obtained in the previous

TABLE III

Comparison of Effects of NaF with Epinephrine and Glucagon on P^{32} Incorporation into Phosphorylase

15 gm. portions of liver slices were incubated for 15 minutes at 37° in 30 ml. of a medium containing 0.15 M NaCl (or NaF) + 0.02 M glycylglycine + 0.001 M phosphate buffer (pH 7.4). 0.045 ml. of 1 per cent epinephrine or 0.66 mg. of glucagon was added at zero time to samples in NaCl. 1 mc. of P^{32} was added after 10 minutes incubation. Purification of phosphorylase with carrier additions and enzymatic inactivation of final preparations are described in the text.

	NaF	Epinephrine	Glucagon
Units LP activity per gm. slice	17.5	14.9	10.4
" " " " mg. protein in final preparation	8.8	10.2	12.9
% slice LP activity recovered	17.8	15.3	15.6
C.p.m. made TCA-soluble after inactivation per % LP recovered	19.2	128	143

experiment (Table I and Fig. 2), these values have been considered an index to the extent of incorporation of P^{32} into phosphorylase.

Although NaF was even more effective than epinephrine and glucagon in maintaining a high level of LP in the slices (compare the first lines of Tables I and III), the incorporation of P^{32} in the presence of NaF was very small (last line, Table III). Epinephrine and glucagon maintained the LP concentration moderately well, but in these cases P^{32} was readily incorporated into the enzyme. This indicated that fluoride inhibited the inactivating enzyme, thus preventing the removal of phosphate from the existing LP and the incorporation of radioactive phosphate into LP. On the other hand, results with epinephrine and glucagon again indicated that they might act by a different mechanism, since P^{32} was readily incorporated into slice phosphorylase in their presence.

Subsequent experiments were compatible with the above interpretation,

as shown by the example in Table IV. The slices were preincubated at 37° for 10 minutes (Flasks 1 and 4 contained NaF-buffer medium and Flasks 2 and 3 NaCl-buffer medium). Flask 2 contained epinephrine from the start of the experiment. After the 10 minute preincubation, the NaCl-buffer medium of Flask 3 was replaced with the NaF-buffer medium containing 1 mc. of P^{32}. At the same time, 1 mc. of P^{32} was added to the other flasks and epinephrine was added to the NaF-buffer medium of Flask 4. Incubation was then continued for an additional $7\frac{1}{2}$ minutes.

TABLE IV

Conditions for Incorporation of P^{32} into Phosphorylase in Presence of NaF

15 gm. portions of slices were incubated for $17\frac{1}{2}$ minutes at 37° in 30 ml. of medium. Flasks 2 and 3 contained 0.15 M NaCl + 0.02 M glycylglycine + 0.001 M phosphate buffer (pH 7.4), while Flasks 1 and 4 contained NaF in place of NaCl. 1 mc. of P^{32} was added to each flask after 10 minutes. 0.045 ml. of 1 per cent epinephrine was added to Flask 2 at zero time and to Flask 4 after 10 minutes incubation. The medium of Flask 3 was decanted at 10 minutes and replaced with 27 ml. of a solution containing 0.15 M NaF + 0.02 M glycylglycine + 0.001 M phosphate buffer. Purification of phosphorylase with carrier additions and enzymatic inactivation of final products are described in the text.

	Flask No.			
	1 (NaF)	2 (epine-phrine)	3 (NaCl-NaF)	4 (NaF + epine-phrine)
Units LP activity per gm. slice.............	10.7	9.0	7.9	11.2
" " " " mg. protein in final preparation.............................	11.2	14.1	14.6	14.1
% slice LP activity recovered...............	5.8	9.5	8.5	11.5
C.p.m. made TCA-soluble after inactivation per % LP recovered.....................	40.0	112	225	29.2

At the end of the incubation period, the media were decanted, the slices homogenized, and the LP purified as in the previous experiment. In Flask 3, the 10 minute preincubation in NaCl-buffer medium caused the LP level to fall (see Fig. 1), and the addition of fluoride for the final $7\frac{1}{2}$ minutes resulted in an increased concentration of LP, as shown by a simultaneous non-radioactive experiment. During this final $7\frac{1}{2}$ minutes, radioactive phosphate was readily incorporated into the phosphorylase protein, as can be seen in the last line of Table IV. It was concluded that dephospho-LP had been formed during the preincubation in NaCl, and on the addition of NaF the reformation of active enzyme by the reactivation (kinase) process proceeded relatively unopposed by the inhibited IE. As this net formation of active enzyme proceeded in the presence of fluoride,

radioactive phosphate was incorporated. Thus it appeared that the presence of dephospho-LP was required for the incorporation of radioactive phosphate into LP.

Conversion of Dephosphophosphorylase to Phosphorylase in Liver Extracts

The incorporation of P^{32} into LP in slices and the stability of the phosphate-protein bond formed pointed strongly to the conclusion that a phosphate transfer system was involved in the conversion of dephospho-LP to LP. The resultant protein-phosphate bond was found to be firm and was not broken by TCA or by short incubation with hot dilute HCl or hot dilute NaOH. In addition, incorporated P^{32} did not exchange upon incubation with P^{31}-orthophosphate (Table II).

Studies on the reactivation process in broken cell preparations led to the finding of a kinase capable of transferring phosphate to dephospho-LP. Incubation of dephospho-LP with diluted dog liver homogenates in the presence of ATP and magnesium ions resulted in the formation of LP. The enzyme catalyzing the phosphate transfer was found to be soluble and was purified about 45-fold by procedures summarized below. 1 unit of phosphokinase was defined as that amount of enzyme which catalyzed the formation of 1 unit of LP in the assay system in 10 minutes at 37°. The specific activity was expressed as units per mg. of protein.

Step 1. Homogenization and Filtration—All steps were carried out in the cold. Dog livers were perfused and homogenized and the homogenates were filtered according to the procedure used for the preparation of filtrates containing LP and IE (3). Phosphokinase remained in the precipitate (filter cake). The specific activity of the homogenate was about 1.5.

Step 2. Washing and Eluting Cake—The filter cake was washed twice with a volume of cold 0.1 M NaF equal to $\frac{2}{3}$ the volume of the homogenate. The phosphokinase remained on the cake during this procedure and was eluted by homogenizing the cake in 300 ml. of cold 0.02 M Tris buffer (pH 9.0) per 100 gm. of liver for 1 minute in a Waring blendor. This suspension was filtered as before and the filter cake was washed with 100 ml. of cold glass-distilled water per 100 gm. of liver. The specific activity in the eluate of the cake was about 4.

Step 3. Freezing and Thawing—It was found that the heavy haze in the eluate of the cake could be removed by freezing, thawing, and subsequent centrifugation. This procedure resulted not only in clarification of the solution, but also in a 2-fold purification of the enzyme.

Step 4. First Ammonium Sulfate—The clarified eluate was brought to 33 per cent saturation with solid $(NH_4)_2SO_4$ and neutralized with KOH. The precipitate was collected by centrifugation and dissolved in 0.04 M Tris buffer (pH 8.0). The specific activity was about 12.

Step 5. Second Ammonium Sulfate—The solution was clarified by centrifugation in the cold at 69,000 × *g* for 30 minutes. The supernatant solution was brought to 30 per cent saturation with a neutralized saturated solution of Versene-treated $(NH_4)_2SO_4$ (3). The precipitate was collected by centrifugation and dissolved in a solution containing 2×10^{-3} M $MgSO_4$ and 5×10^{-2} M Tris (pH 8.0).

Step 6. Dialysis and $Ca_3(PO_4)_2$ Gel—The solution was dialyzed for 2 hours *versus* alkalinized glass-distilled water and then diluted with 0.01 M Tris buffer (pH 7.2) to a protein concentration of 10 mg. per ml. To the diluted, dialyzed solution was added 0.1 volume of $Ca_3(PO_4)_2$ gel. Most of the phosphokinase activity was adsorbed on the gel and subsequently eluted with a solution containing 0.3 M Na_2SO_4 and 0.05 M Tris buffer (pH 8.0) in a volume equal to one-half that of the diluted solution. The specific activity of the eluate was about 40.

Step 7. Third Ammonium Sulfate—The eluate was brought to 30 per cent saturation with a neutralized, saturated solution of Versene-treated $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in a solution containing 2.5×10^{-3} M $MgSO_4$ and 0.05 M Tris buffer (pH 8.0). The specific activity of this fraction was about 70. Approximately 10 per cent of the phosphokinase present in the liver homogenate was recovered by these procedures.

Requirement for ATP and Mg^{++}—The requirement of phosphokinase for commercial ATP and Mg^{++} was demonstrated even in homogenates when diluted for assay. Manganous and calcium ions failed to replace the Mg^{++} requirement of the crude or partially purified enzyme. With highly purified enzyme, uridine triphosphate, inosine triphosphate, cytidine triphosphate, adenosine diphosphate, and adenosine-5-phosphate were tested for their ability to replace commercial samples of ATP in the phosphokinase assay system with negative results. The term "commercial ATP" is used, since the participation of ATP as the actual phosphate donor has not been absolutely proved; this point is currently under investigation.

LP Formation—The product of the phosphokinase reaction (LP) was identifiable through its enzymatic activity. However, additional proof that restoration of enzymatic activity was accompanied by phosphate transfer to dephospho-LP was sought. Two aliquots of dialyzed dephospho-LP were incubated for 80 minutes at 27° in a solution containing (at final concentrations) 5×10^{-4} M ATP, 2×10^{-2} M NaF, 2.5×10^{-3} M $MgSO_4$, and 1.5 mg. per ml. of dephospho-LP. Highly purified phosphokinase (third $(NH_4)_2SO_4$ fraction, dialyzed for $1\frac{1}{2}$ hours) was added to one aliquot at the beginning of the incubation period and to the other at the end of the incubation which was terminated by the addition of 2 volumes of a solution of saturated $(NH_4)_2SO_4$. In the presence of phosphokinase

80 per cent reactivation of dephospho-LP[3] occurred. Both samples were precipitated twice with $(NH_4)_2SO_4$, dialyzed for 2 hours, and precipitated with alcohol with 69 per cent recovery of the reactivated LP. Both samples were then incubated with IE for 45 minutes. Aliquots of each sample were fixed with TCA at the beginning and at the end of the incubation, and the TCA-supernatant solutions were analyzed for inorganic phosphate, as shown in Table V. At the end of incubation all the LP had been returned to the dephospho form and approximately 0.27 γ of phosphorus appeared in the TCA-supernatant fluid for every mg. of LP

TABLE V

Phosphate Transfer to Dephospho-LP Catalyzed by Phosphokinase

Samples of dephospho-LP, ATP, and Mg^{++} were incubated with and without phosphokinase. Following ammonium sulfate and alcohol precipitation, the samples were incubated with IE. At zero time and at 45 minutes, aliquots were placed in TCA; after centrifugation, the TCA-supernatant solutions were analyzed for inorganic phosphate. Other aliquots were assayed for LP activity.

History of sample	Time of incubation with IE	Composition of sample		P released by IE into TCA-supernatant fluid
		Dephospho-LP	Active LP	
	min.	*mg.*	*mg.*	γ
Incubated with phosphokinase	0	0.25	1.00	
" " "	45	1.25	0	0.27
" without phosphokinase....................	0	1.25	0	
Incubated without phosphokinase....	45	1.25	0	0

nactivated. This value agrees well with those observed during the inactivation of "native" LP (2). In the sample which had not been incubated with phosphokinase, *no increase in TCA-soluble phosphate* was detected after incubation with IE.

DISCUSSION

The concentration of active phosphorylase in liver tissue is the result of a balance between two opposing reactions: the inactivation of the enzyme by phosphorylase phosphatase (IE) and the reactivation of the resulting dephosphophosphorylase by the phosphokinase system. Sodium fluoride has been shown to decrease the rate of formation of dephosphophosphorylase by inhibiting IE, thereby allowing the constantly operating

[3] This sample, as well as those described under "Methods," could be reactivated completely under appropriate conditions.

phosphokinase system to bring about a net increase in LP concentration. Epinephrine and glucagon may stimulate some portion of the phosphokinase system and thus cause a net increase in LP concentration. Although the evidence to date is compatible with this possibility, alternative explanations will be considered and evaluated in the future.

Phosphokinase activity measured in diluted homogenates was sufficient to implicate the enzyme in the rapid reactivation of phosphorylase which occurs when epinephrine or glucagon is added to slices. It was calculated from the specific activity of phosphokinase (1.5) and of phosphorylase (0.13) in homogenates that dog liver contains enough of the phosphokinase to reactivate all the phosphorylase present in 1 minute at 37°. As yet, the effects of glucagon or epinephrine on the phosphokinase system in broken cell preparations have been variable. The detailed mechanism of action of these agents is under investigation. Preliminary experiments have shown that inhibitory agents or processes are operating on the phosphokinase reaction in concentrated, cell-free homogenates; therefore numerous factors enter into the analysis of hormone action in concentrated tissue preparations.[4]

During the progress of this work, Fischer and Krebs (7) reported the presence of an enzyme in rabbit muscle extracts which catalyzed the conversion of rabbit muscle phosphorylase b to phosphorylase a in the presence of ATP and manganous ions. The relation of this enzyme to the magnesium-requiring liver phosphokinase is uncertain. The differences between the conversion of liver phosphorylase to dephospho-LP by IE and the conversion of rabbit muscle phosphorylase a to phosphorylase b by phosphorylase-rupturing enzyme have been noted previously (2). However, dog heart muscle contains enzymes capable of inactivating liver phosphorylase and reactivating liver dephosphophosphorylase. These enzymes, as well as heart phosphorylase, have been purified to varying degrees in this laboratory. Highly purified IE from heart appears to be similar to IE from liver, and partially purified phosphokinase from heart (acting on liver dephosphophosphorylase) requires ATP and Mg^{++} as does the liver phosphokinase. The relationship of these enzyme systems in liver and in heart is currently under investigation.

SUMMARY

1. Radioactive phosphate was rapidly incorporated into liver slice

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[4] Homogenates prepared from slices preincubated at 37° responded consistently to the addition of epinephrine or glucagon, as indicated by a marked increase in the concentration of LP in these homogenates. Additions of magnesium ions and ATP were important in the demonstration of large responses of the homogenates to the hormones. Details of these experiments will be presented in a subsequent publication.

phosphorylase. Epinephrine and glucagon greatly increased this incorporation.

2. The protein-phosphate bond appeared to be a covalent bond and did not exchange with phosphate as the enzyme acted catalytically.

3. Phosphate transfer to dephosphophosphorylase was studied in broken cell preparations. An enzyme was found capable of converting enzymatically inactivated phosphorylase to active phosphorylase in the presence of adenosine triphosphate and magnesium ions.

4. The enzyme, liver dephosphophosphorylase kinase, has been purified about 45-fold. The enzymatic reactivation of purified dephosphophosphorylase catalyzed by this purified enzyme has been described. The phosphorylase formed by reactivation was shown to contain phosphate which could be removed on incubation with the inactivating enzyme.

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SIMPLE MICRODETERMINATION OF URIC ACID

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In 1947 Kalckar introduced a comprehensive differential spectrophotometric system for the analysis of purines, including uric acid (1). Praetorius and his associate Poulsen employed this procedure and have published detailed directions for a practical uric acid method (2, 3).

During the past 4 years, over 2000 routine uric acid determinations on blood and urine have been performed in this laboratory by a modification of the Kalckar approach. This modification, while similar to that of Praetorius, provides some features that the present authors feel are more convenient and require less working time. In particular, by use of a more dilute uricase preparation, uric acid destruction proceeds slowly enough to enable the technician to make a single, reliable reading of initial absorbance; he need not record a series of rapidly decreasing absorbances, with final extrapolation back to the initial absorbance.

For nearly all samples of urine and serum, determination can be completed within 2 to 4 hours. If desired, however, incubation may be allowed to continue overnight. Experimental evidence is presented that reliable results are obtained, even when an overnight incubation period is employed.

A particular advantage of the Kalckar procedure is that it requires no dangerous reagents such as cyanide, which unfortunately is commonly used today in colorimetric methods.

Principle

Uric acid strongly absorbs ultraviolet light of wave-length 292 mμ (Fig. 1) and is specifically and completely converted to non-absorbing products by action of the enzyme uricase. Therefore, the decrease in absorbance which occurs at 292 mμ upon incubation of uricase is a direct measure of the uric acid content of a solution.

Materials and Method

Special Equipment—Beckman model DU spectrophotometer (No. 37623), ultraviolet attachment (No. 37002), two matched silica cells (1.0 cm. light path), one Corex cell (optional). Substitute glass cells, if necessary, and use wider slit widths on the spectrophotometer.

Culture tubes, 16 × 100 mm., with racks (for incubation).

Cups, about 1 cm. in diameter with suspension hooks (to receive 50 to 200 µl. samples). A simple cup (Fig. 3) can be made by heating and flattening the base of a 1 cm. (outside diameter) test-tube, cutting to about 7 mm. in length, winding a length of corrosion-resistant wire (about 25 gage) around its circumference, and twisting and bending the ends of the wire into a hook. Avoid materials that would contaminate the solution.

Micro pipettes, Kirk type (4), 50, 100, and 200 µl. for pipetting samples.

Pipette, Krogh-Keys syringe type (5), 10 ml., adjusted to deliver 9.9 ml. of uricase reagent.
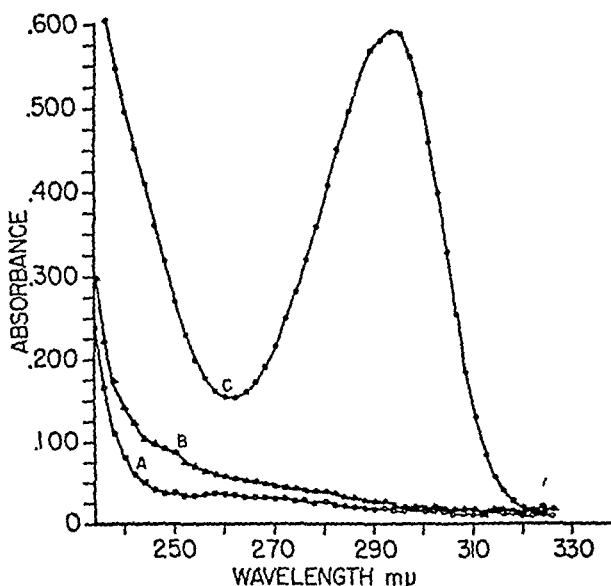
FIG. 1. Ultraviolet absorption spectra. Curve A, glycine buffer, 0.1 M, pH 9.4; Curve B, buffer plus Worthington uricase, 6 mg. per liter; Curve C, buffer plus uric acid, 8 γ per ml. The slit width was progressively increased from 0.13 mm. (325 mµ) to 0.36 mm. (235 mµ).

Beckman model G pH meter.

Water bath regulated near 45° (not critical).

Small cylinder of oxygen.

A deep freeze or equivalent is desirable for storage of frozen samples awaiting analysis.

Reagents—

5 N NaOH.

Glycine buffer (0.1 M). Dissolve 7.5 gm. of glycine in about 800 ml. of distilled water; adjust to pH 9.4 ± 0.1 with 5 N NaOH (approximately 8 ml.); dilute to 1 liter.

Routine uric acid standard (400 γ per ml.). Dissolve 40.0 mg. of uric acid (preferably recrystallized and dried) in 100 ml. of glycine buffer. If aliquots are frozen in small vials, they remain stable indefinitely.

Uricase reagent. Dissolve 40 mg. of dry uricase[1] (Worthington Biochemical Corporation, Freehold, New Jersey) in 1 liter of glycine buffer. Store in the refrigerator.

The activity of this reagent may vary over a 10-fold range and still give satisfactory results. Nevertheless, it is desirable to check the approximate activity of each new lot of uricase by a preliminary standard run. Pipette 100 μl. of routine uric acid standard (400 γ per ml.) into 9.9 ml. of the reagent, mix, and allow to incubate at room temperature. Measure the absorbance decrease at 292 mμ for the first 10, 20, or 30 minutes. If the rate of decrease is not within the range of 0.0003 to 0.003 absorbance unit per minute, adjust it accordingly by either proportional dilution with more glycine buffer or proportional addition of more uricase.[2]

If necessary to complete the determination within 2 to 4 hours, adjustment should be made near the maximal rate (0.003). This rate has been chosen as the fastest consistent with obtaining a single, reliable reading of initial absorbance (allowable error of 0.001 absorbance unit).

Absorbance Measurements—For routine uric acid determinations, set the Beckman spectrophotometer as follows: wave-length 292 mμ, selector switch 0.1, slit width 0.17 mm., and sensitivity knob approximately three turns from the clockwise position.

In so far as possible, initial absorbance should be held in the range of minimal instrumental error, approximately 0.200 to 0.800. If the initial absorbance is too high, as may occur with serum, a denser (Corex or glass) reference cell can be used to improve precision, although this is unnecessary for purposes of clinical diagnosis. Since absorbances are strictly additive, a sample that reads 0.900 against a silica reference will read 0.500 (0.900 minus 0.400) against a Corex reference which itself reads 0.400 against the silica reference. Praetorius and Poulsen (3) discuss this matter.

Standardization—Dilute 5 ml. of routine uric acid standard to 200 ml. with glycine buffer. Pipette aliquots of 0, 0.5, 1, 2, 4, 6, 8, and 10 ml., and dilute each to 10 ml. with glycine buffer. Determine the absorbance, and plot a standard curve of absorbance *versus* concentration (Fig. 2). This standard curve is both (1) a straight line (follows Beer's law) over the en-

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[1] Worthington now offers a more concentrated uricase in aqueous suspension. The preparation has not yet been used in this laboratory.

[2] This laboratory has had very good results with the economical Worthington product. Uricase Ido (Ferrosan Export Corporation, Copenhagen, Denmark) is an even purer product, excellent but somewhat more expensive per unit of activity. (Dilute 0.4 ml. of the commercial suspension with 1 liter of glycine buffer and proceed.) Delta uricase (Delta Chemical Works, New York) is very crude and requires incubation overnight, but has given adequate results in much past work here. (Suspend 0.5 gm. in 1 liter of glycine buffer, let stand 3 hours, remove the coarse particles by filtration, and proceed.)

tire applicable range and (2) is reproducible over an extended period of time.

Uric Acid Determination[3] *(Fig. 3) in Urine*—Pipette 9.9 ml. of uricase reagent into each of ten to twenty culture tubes. Hook a cup over the side of each tube. Pipette 100 μl. of distilled water into the first cup (for a reagent blank), 100 μl. of the routine uric acid standard into the second cup, and 100 μl. of sample into each of the other cups. If the samples are pipetted directly into the reagent tubes, premature uricase action may cause low values. After the entire series has been set up, slip each cup into its culture tube, stopper the tube, shake thoroughly, promptly transfer
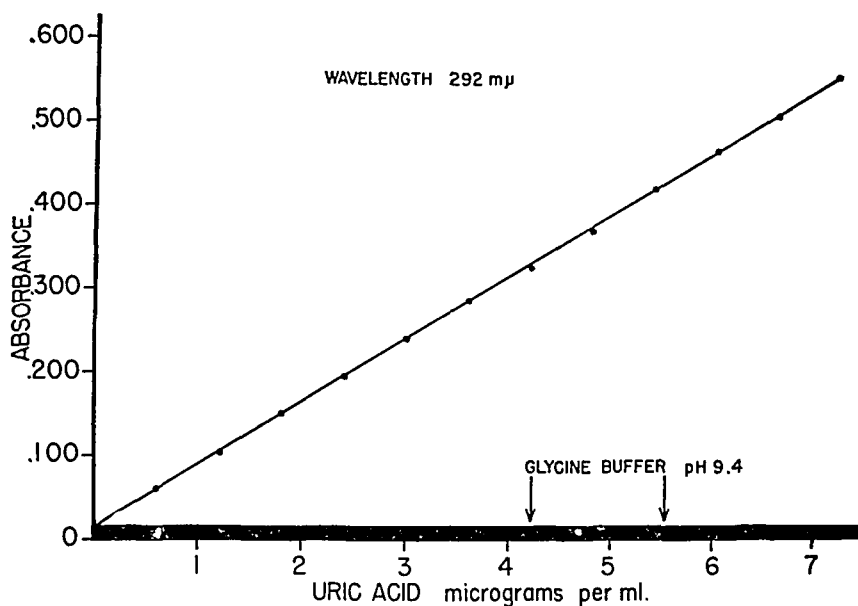
FIG. 2. Standard curve. Uric acid in glycine buffer, 0.1 M, pH 9.4; wave-length 292 mμ. Glycine buffer blank = 0.018.

a portion to the silica cell, and read the initial absorbance (A_0). Mark the meniscus of the remaining solution with a wax pencil. (Dixon Phano No. 80 for glazed surfaces will not come off in the warm water bath.) Place the open tubes in the open 45° water bath. Oxygenate the contents of each tube for 10 to 20 seconds by passing a slow flow of oxygen from a cylinder through a constricted glass tube (Wintrobe pipette), inserted vertically with its tip close enough to agitate the solution without spattering. Allow the samples to incubate 4 to 16 hours. Replenish evaporated water

[3] Since uric acid destruction can occur rapidly in biological samples, store the samples no longer than 3 hours at room temperature or 3 days in the refrigerator. Frozen samples (deep freeze) are stable indefinitely. To avoid possible uric acid precipitation in urine, add 10 ml. of 5 per cent NaOH to the bottle in which a 24 hour urine specimen is to be collected.

by restoring the initial meniscus of each tube with distilled water (several drops), mix thoroughly, and read the final absorbance (A_f).

Uric Acid Determination in Serum—Follow the same procedure as for urine except that incubation should be carried out from 2 to 16 hours at room temperature. Turbidity frequently develops in serum samples incubated overnight at 45°.

Calculations—Subtract the final absorbance from the initial absorbance. Correct this absorbance change, if necessary, by adding any absorbance increase shown by the uricase reagent blank. Divide the corrected absorb-

A   B
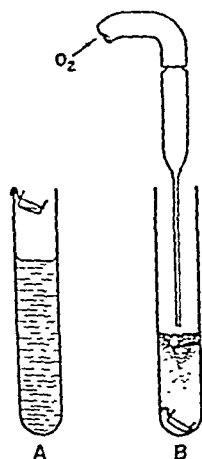
FIG. 3. Two stages of the procedure. *A*, the suspended cup, containing 100 μl. of sample, is ready to be slipped into 9.9 ml. of uricase reagent, immediately prior to mixing and transfer of an aliquot of the mixed solution to the optical cell for initial absorbance measurement. *B*, the remaining solution is being oxygenated prior to incubation.

ance change by the factor k to give the uric acid concentration (micrograms per ml.) in the diluted sample. The factor k is the slope of the standard curve (absorbance change ÷ concentration change) and will have a value near 0.075. Typical calculations are illustrated in Table I.

Important Precautions—(1) Owing to the wide variation in properties among different uricase preparations, use each new preparation with discretion. Discard any uricase reagent (prepared as specified under "Uricase reagent") if the absorbance of its reagent blank (1) is initially greater than 0.200 or (2) increases by more than 0.010 upon 45° incubation overnight. (2) Avoid water contaminated with ultraviolet-absorbing material. Once in this laboratory, a high and variable absorbance was traced to accidental contamination of the distilled water by an old rubber dispensing tube. (3) Use strictly clean glassware well rinsed with distilled water.

One series of erratic determinations was traced to a batch of culture tubes which, although visibly clean, was found to give water blanks differing by as much as 0.025 absorbance unit, owing presumably to residual detergent. (4) Avoid the presence of solvent or other vapors during uricase incubation. While some (including chloroform) apparently will not interfere, others (including ether) may inhibit the enzyme action significantly.

Results

The reliability of the new procedure has been demonstrated by the following experiments.

Each of eleven samples was analyzed in duplicate (Worthington uricase), once under the fast rate conditions of Praetorius and Poulsen (2 hours) and

TABLE I

Typical Calculations

Sample	Absorbance				Uric acid concentration	
	A_0, initial	A_f, final	$A_0 - A_f$, change	$A_0 - A_f$,* corrected for blank	Diluted sample†	Original sample
					γ per ml.	γ per ml.
Serum....................	0.566	0.536	0.030	0.033	0.44	44‡
Urine....................	0.447	0.287	0.160	0.163	2.17	217

* The uricase reagent blank increased 0.003 upon incubation (from 0.141 to 0.144).
† 0.1 ml. of sample was added to 9.9 ml. of uricase reagent (dilution 1 to 100).
‡ Uric acid concentration in original sample $= 0.033/k \times 100 = 44$ γ per ml. $(k = 0.075)$.

once under the slow rate conditions of the present method (16 hours). Good agreement was found: For five urine samples with a mean absorbance decrease of 0.212, δ (standard deviation) was 0.006. For six serum samples with a mean absorbance decrease of 0.042, δ was 0.005 (0.002 with omission of one erratic value).

Sixteen additional samples were analyzed under fast rate conditions (Worthington uricase). The absorbance decreases attained within 2 hours were little changed by prolonging incubation to 16 hours (with one exception). For nine urine samples with a mean absorbance decrease of 0.265, the mean absorbance error (due to prolonged incubation) was 0.0015, δ 0.0019 (one sample with an error of 0.021 excluded). For seven serum samples with a mean absorbance decrease of 0.055, the mean absorbance error was 0.0020, δ 0.0016.

Several experiments showed that incubation of serum at 45° often caused turbidity, especially when Delta uricase was used. Incubation at 25° avoided this difficulty.

Precision absorbance data, all obtained with the crude Delta uricase, give δ 0.0008 for eighteen determinations on four urine samples; δ 0.0011 for seventeen determinations on three serum samples. This precision, essentially that of photometric measurement alone (balancing and reading the spectrophotometer), represents the potentialities of the method as performed under favorable conditions. Day to day precision, introducing other errors, will always be somewhat less.

Recoveries (nine experiments) of 1 to 2 γ per ml. of uric acid added to diluted urine and serum samples (initial concentrations of 0.5 to 5.5 γ per ml.) varied from 95 to 104 per cent; mean 100.4 per cent, δ 2.6 per cent.

Normal values, obtained by the present method for ten normal subjects on a low purine diet, are as follows: urine 583 $\pm$ 81 (standard deviation) mg. per 24 hours; serum 3.64 $\pm$ 0.68 mg. per 100 ml.

DISCUSSION

Colorimetric Methods—Uric acid methods now in common use are based on colorimetric measurement of the blue color formed when uric acid reduces phosphotungstate (Folin and Wu (6)) or arsenotungstate (Benedict (7)), usually with color enhancement by urea-stabilized cyanide. Many workers in the past, including Folin and Benedict, have demonstrated that these methods are non-specific and unreliable (8). Many natural substances other than uric acid give the blue color, and the attempted removal of these substances by precipitation techniques is subject to serious manipulative loss. Although uricase incubation can solve this problem satisfactorily, it remains entirely ineffective against a second type of interference from substances which, although non-chromogenic themselves, interact with uric acid and the reagents to enhance or depress the initial color development. Even the basic color development reactions with pure uric acid are beset with difficulties, as has been well documented by Folin himself (9). Sensitive to many experimental conditions, these reactions give a variable and non-linear relationship between absorbance and uric acid concentration.

These objections are avoided by the fundamentally new approach of Kalckar, as applied also in the method of Praetorius and Poulsen and the present method. The particular advantage of the present procedure is the need for only one initial absorbance reading. Also uricase and glycine buffer are combined in one reagent to which the sample is added, and simple uric acid standard solutions in buffer are substituted for more complex lithium urate standards.

The Praetorius method is faster in the sense that one can complete a determination within $\frac{1}{2}$ to 2 hours, whereas the present method requires 2 to 4 hours. However, the present method is faster in the sense that an average of fifteen determinations can be performed per analyst hour of

working time, compared to five determinations per analyst hour by the Praetorius method (2). Since uric acid is not an "emergency" determination, the more leisurely working pace, yet reduced working time, of the present method may be preferred in many laboratories, especially when many determinations are performed together.

The present method, in essentially its present form, has supplied the extensive data for two clinical uric acid studies in this laboratory[4] (10).

SUMMARY

Uric acid in urine and serum is determined by a modification of the Kalckar procedure, by utilizing the specific decrease of uric acid absorbance at 292 mμ in the presence of uricase.

The new method, by providing fifteen determinations per analyst hour, requires one-third the working time of the method of Praetorius and Poulsen. This advantage results from use of a uricase preparation dilute enough to permit a single, reliable reading of initial absorbance, instead of requiring extrapolation back from several rapidly decreasing readings. Although a determination can be completed in 2 to 4 hours, good precision and accuracy are found even when samples are allowed to incubate overnight (16 hours).

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α-KETOGLUTARIC DEHYDROGENASE

V. GUANOSINE DIPHOSPHATE IN COUPLED PHOSPHORYLATION*

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The phosphorylation of ADP by inorganic phosphate coupled to the oxidation of KG has been described previously (1, 2).[1] The reactions leading to the phosphorylation are shown in Equations 1 to 3. We have

$$KG + DPN^+ + CoASH \rightarrow succinyl\ S\text{-}CoA + DPNH + H^+ + CO_2 \quad (1)$$

$$Succinyl\ S\text{-}CoA + ADP + P_i \rightleftharpoons succinate + CoASH + ATP \quad (2)$$

$$Sum.\ \ KG + DPN^+ + ADP + P_i \rightarrow succinate + DPNH + ATP + H^+ + CO_2 \quad (3)$$
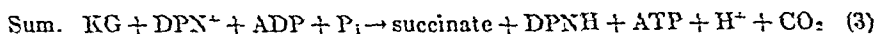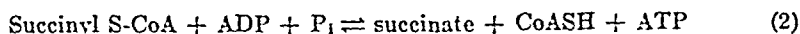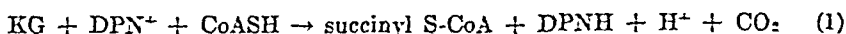
reported in a series of preliminary communications that in animal tissues Equation 2 can be further separated into two steps (Equations 4 and 5) and requires the presence of GDP as cofactor (3–5). The present paper

$$Succinyl\ S\text{-}CoA + GDP + P_i \rightleftharpoons succinate + CoASH + GTP \quad (4)$$

$$GTP + ADP \rightleftharpoons GDP + ATP \quad (5)$$

describes the purification of the two enzymes participating in the phosphorylation of ADP and deals with the analysis of the two reactions.

The enzyme catalyzing Equation 4 will be referred to as the GDP phosphorylation enzyme (or phosphorylation enzyme). The enzyme catalyz-

[1] The following abbreviations are used: α-ketoglutarate, KG; diphosphopyridine nucleotide, DPN; coenzyme A, CoA or CoASH; adenosine-5'-mono-, di-, and triphosphates, A-5'-P, ADP, and ATP, respectively; guanosine (G), inosine (I), uridine (U), and cytidine (C) phosphates similar to adenine nucleotides above; inorganic phosphate, P_i; tris(hydroxymethyl)aminomethane, Tris; A for absorbancy or log I_0/I.

ing the transphosphorylation is a nucleoside diphosphokinase (NDP kinase) (6–8).

Results

Initial Observations

Some of our early experiments which indicated that two different enzymes are required for the succinyl CoA-linked phosphorylation of ADP (Equation 2) are reported in Table I.[2] When the preparations were tested individually in the coupled phosphorylation system (Equation 3), negligible activity was found. However, when the two preparations were

TABLE I

Requirement of Two Enzymes for ADP Phosphorylation Reaction

Enzyme source	DPNH assay		CoASH assay	
	Protein	ΔA per min.	Protein	$-\Delta$ —SH
	mg.		*mg.*	*μmole*
Kidney	0.17	0.006	0.04	0.014
Heart	0.85	0.000	0.55	0.000
Mixture	0.17 + 0.85	0.142	0.04 + 0.55	0.180

The DPNH assay is carried out spectrophotometrically as described previously (1). For the CoASH assay, reduced CoA (0.32 μmole), KBH₄ (1 μmole), succinate (10 μmoles), ATP (1 μmole), and Tris (3 μmoles) in 0.25 ml. at pH 7.5 were incubated for 10 minutes at 30°. The pig kidney enzyme was extracted from acetone powder and fractionated essentially as described in the text. The pig heart enzyme was extracted by homogenizing minced heart in phosphate buffer, and the supernatant solution obtained after precipitation at pH 5.4 (10) was allowed to stand overnight at the acid pH. The fraction between the limits of 0.48 and 0.57 saturation of ammonium sulfate was used after dialysis.

combined, the expected high activity was observed. Both of these preparations were again necessary for the reaction when studied in the reverse direction by measuring CoASH disappearance in the presence of ATP and succinate (Equations 5 and 4 from right to left).[3] The results are presented in Table I.

[2] The data for Table I were obtained in collaboration with Dr. L. Ouellet. The authors are grateful to Dr. D. Goldman for supplying these enzyme preparations obtained during the course of his studies on the acetoacetate cleavage reaction (9). Both the phosphorylation enzyme and the kinase were present in the heart preparation used in previous studies (1, 2). This, as well as the need for GDP in the system, is seen in Fig. 1, Curve 3.

[3] The CoASH disappearance assay is an extremely sensitive test for contamination of adenine nucleotides with the cofactor (GDP or GTP). We found 0.1 per cent GDP (or GTP) impurity in a commercial sam

Purification of Enzymes

Pig kidney cortex is a convenient source for the GDP phosphorylation enzyme, since the NDP kinase activity is low in this tissue. The enzyme is also present in liver and heart muscle. The NDP kinase can be purified readily either from heart muscle or from kidney cortex, since it is considerably more stable to acid and to heat than the phosphorylation enzyme.

GDP Phosphorylation Enzyme—The enzyme is assayed in terms of the rate of DPN reduction by coupling with the KG dehydrogenase system. The conditions for the assay are as described previously (1), except that 0.05 µmole of GDP was added instead of ADP (see Fig. 2). The rate of

TABLE II

Cofactor Requirement for Synthesis of Succinyl CoA

ATP	CoASH	Cofactor	$-\Delta-SH$
µmole	*µmole*	*µmole*	*µmole*
0.50	0.32		0.01
0.50	0.32	0.0015	0.11
0.50	0.32	0.006	0.18
0.50	0.47*		0.18
0.50†	0.32		0.14

The reaction mixture consisting of KBH_4 (1 µmole), $MgCl_2$ (2 µmoles), Tris (6 µmoles), succinate (20 µmoles), GDP phosphorylation enzyme (0.1 mg.), NDP kinase (0.025 mg.), and the above components, as specified, in 0.5 ml. at pH 7.5 was incubated for 5 minutes. The sulfhydryl group of CoASH was determined by the nitroprusside reaction.

* CoA before reprecipitation.

† Commercial ATP before further purification.

DPN reduction was followed in the recording Beckman spectrophotometer at 340 mµ for 1 minute. The enzyme was usually assayed at two or three levels. 1 unit of activity is the amount of enzyme that produces a change in absorbancy ($\log I_0/I$) of 1.0 per minute at 30° under standard conditions. Specific activity is expressed as units per mg. of protein.

Pig kidney cortex was diced into $\frac{1}{4}$ inch cubes and comminuted in lots of 400 gm. with 1.6 liters of cold 0.9 per cent KCl in a high speed blender for 40 seconds. During the blending 1 to 3 ml. of 6 N KOH was added to

It is of interest to note that the level of the contaminant was below the limits of detection of the method before crystallization. Apparently the impurity had been concentrated in the crystals either because of cocrystallization or the lower solubility of guanosine derivatives compared to adenine derivatives. It may be pointed out that the crystalline nucleotides may also be contaminated with unspecified amounts of amorphous material, indicating the need for caution in interpreting data obtained even with crystalline nucleotides.

maintain the pH around 7.5. The homogenate was centrifuged at 1900 × g for 10 minutes and the supernatant fluid discarded. The residue was washed by suspending in 4.5 liters of cold 0.9 per cent KCl and collected in a refrigerated Sharples centrifuge. Acetone powder was prepared by conventional methods from the residue after suspension in 500 ml. of KCl solution. The yield was approximately 250 gm. from 4 kilos of kidney cortex.

The acetone powder (100 gm.) was extracted by continuous stirring with 1 liter of cold 0.02 M KHCO₃ for 20 minutes. The temperature rose to 15° during the extraction. All subsequent operations were at 5° or lower, as indicated. A clear supernatant solution was obtained by centrifuging for 1 hour at 2500 × g (yield 3500 units; specific activity 0.35). Ammonium sulfate (30 gm. per 100 ml.) was added slowly, and the mixture was stirred for 15 minutes. More ammonium sulfate was dissolved in the supernatant fluid obtained after centrifuging for 1 hour (20 gm. per 100 ml.). The precipitate was collected as before, dissolved in about 20 ml. of 0.02 M phosphate buffer at pH 7.0, and dialyzed against the same buffer for 12 hours (yield 3100 units; specific activity 1.5). The solution was diluted to a protein concentration of approximately 20 mg. per ml., and the pH was brought to 5.9 with acetic acid. The solution was then fractionated with ethanol immediately. The precipitate formed on addition of 33 ml. of 50 per cent alcohol (volume per volume) per 100 ml. of enzyme solution (0° to −3°) was removed by centrifugation for 1 hour at 2500 × g. For every 100 ml. of supernatant solution 109 ml. of 50 per cent ethanol (by volume) were added slowly. The solution was allowed to cool to −14° during this addition and centrifuged at the same temperature for 1 hour. It was more important to control the temperature carefully at this stage, since the enzyme redissolved appreciably at higher temperatures. The precipitate was taken up in 0.02 M phosphate, pH 7.0, and dialyzed for 12 hours (yield 2400 units; specific activity 3.0). The preparation was stable for several months when kept frozen. The purification over the acetone powder extract varied from 10- to 20-fold, depending on the specific activity of the initial extract. The final yield and activity were consistently reproducible. At this stage of purification the enzyme preparation was essentially free of the NDP kinase, but contained an active phosphatase specific for GDP, IDP, and UDP (11).

NDP Kinase—The assay involves coupling the kinase with the KG dehydrogenase and GDP phosphorylation enzyme systems (see the section above) and measuring the continued rate of DPN reduction after the final addition of ADP. In addition to the components described before, the reaction mixture contained the GDP phosphorylation enzyme (0.3 to 0.4 unit) and GDP (0.02 μmole). The final addition of ADP (2 μmoles) was made when the system had reached the state of slow and steady DPN

reduction (see Fig. 2). The assay was generally carried out at three levels of enzyme, and the activity was calculated from the slope of the plot of enzyme concentration against rate of DPN reduction (1).

Acetone powder was prepared by conventional methods from beef heart mitochondria (12). The yield of acetone powder was 60 to 80 gm. per kilo of wet minced heart muscle. In a typical fractionation, the acetone powder (44 gm.) was extracted with 10 volumes of 0.02 M phosphate buffer at pH 7.0 for 20 minutes and centrifuged for 1 hour at 2500 × g. This and all other operations were carried out between 0° and 5°. The activity in the clear extract was 170 units, specific activity 0.15. Ammonium sulfate (76.5 gm.) was dissolved in the extract (353 ml.), and, after 15 minutes, the precipitate was removed by centrifugation. More ammonium sulfate (62 gm.) was dissolved in the supernatant solution (340 ml.) and the precipitated protein recovered. It was dissolved in 0.02 M phosphate, pH 7.5, and dialyzed against the same buffer for 18 hours (yield 140 units; specific activity 0.26). The enzyme solution was diluted with 0.02 M phosphate, pH 7.5, to a protein concentration of 20 mg. per ml., and 1 M $MgCl_2$ was added to a final concentration of 0.01 M. The solution was immersed in a water bath at 80° and stirred vigorously. The temperature rose to 53° in less than a minute. The solution was maintained at that temperature for 2 minutes and then cooled quickly in an ice bath. The heating conditions are critical, since a lower temperature does not result in complete inactivation of the GDP phosphorylation enzyme, and higher temperatures produce appreciable loss in NDP kinase activity. The denatured protein was removed by centrifugation. The supernatant solution (24 ml.) contained 125 units of enzyme of specific activity 0.69. The protein precipitated by the addition of 6.7 gm. of ammonium sulfate was removed. Further addition of 0.8 gm. of ammonium sulfate to 22.5 ml. of supernatant solution precipitated the enzyme. It was dissolved in and dialyzed against 0.02 M phosphate, pH 7.5, for 12 hours. The final yield was 105 units of enzyme, specific activity 2.5. The purification was 17-fold over the initial acetone powder extract, with a yield of 62 per cent. Approximately the same amount of enzyme was extracted if an acetone powder was made from whole heart. However, the specific activity of the enzyme in the first extraction was generally less than one-third of that obtained in an extract of mitochondrial acetone powder. The preparation at this stage was completely free of succinyl CoA deacylase and of the GDP phosphorylation enzyme. It was stable for several months at −10°.

Cofactor

The evidence presented in preliminary communications (3–5) suggested to us that commercial samples of ADP contained an impurity which was actually a cofactor for the phosphorylation of ADP. In order to test this

possibility a sample of ADP was purified by chromatography on paper by use of the isobutyric acid-ammonium isobutyrate system (13). Five spots were obtained (R_F 0.49, 0.38, 0.29, 0.21, and 0.17, respectively), of which the three fastest moving were the mono-, di-, and triphosphates of adenosine. The ADP (R_F 0.38) recovered from the paper was completely inactive in the system with either or both enzymes, but the activity was restored by the addition of the eluate from the spot below ATP (R_F 0.21) (Fig. 1, Curve
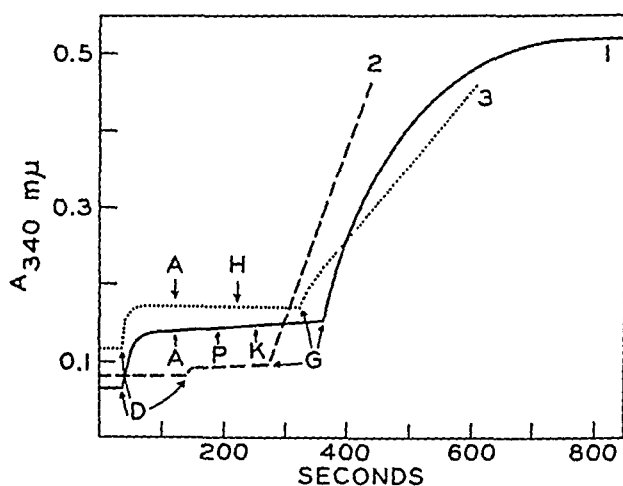
FIG. 1. Requirement for an additional cofactor in the phosphorylation of ADP coupled to α-ketoglutarate oxidation. The reaction mixture consisted of KG (5 μmoles), DPN (1 μmole), CoA (0.02 or 0.10 mg.), cysteine (10 μmoles), MgCl₂ (10 μmoles), and phosphate (50 μmoles) in 3.0 ml. at pH 7.2. D refers to KG dehydrogenase, A to ADP (repurified), P to 0.1 mg. of GDP phosphorylation enzyme from kidney, K to 0.1 mg. of kinase from heart muscle, H to 2 mg. of a preparation from heart muscle (2) containing both P and K above, and G to the cofactor preparation. For Curve 1, G was a 10 γ sample isolated from commercial ADP; for Curve 2, a sample (1 mg.) obtained from yeast extract by adsorption on charcoal; for Curve 3, 0.03 μmole of purified GDP. For Curve 2, ADP (2 μmoles), P, and K were added initially to the reaction mixture. The absorbancy was followed in the Beckman DUR spectrophotometer.

1). The requirement for the same cofactor for the synthesis of succinyl CoA from succinate was examined with rigorously purified preparations of ATP and CoA. Again, it was found that the reaction did not proceed unless supplemented with the cofactor (Table II). The data also indicate that commercial samples of ATP and some samples of CoA prepared by the method of Beinert et al. (14) are contaminated with the cofactor (guanosine nucleotides).

The absorption spectrum of the cofactor obtained from the paper chromatogram was similar to that of guanosine. The addition of the cofactor and the enzyme preparation from pork kidney to the KG dehydrogenase system produced an increase in DPNH equivalent to the guanosine con-

tent (calculated from the absorption at 260 mμ). A rapid, stoichiometric assay for the cofactor was suggested by this observation.

A concentrate of nucleotides from yeast extract prepared by adsorption on charcoal (14) contained significant cofactor activity (Fig. 1, Curve 2). The isolation of the cofactor with the aid of the assay mentioned above and its identification with GDP are described in the following communication (15).

Reaction Sequence

The different steps involved in the phosphorylation of ADP coupled to KG oxidation are demonstrated spectrophotometrically in Fig. 2, Curve
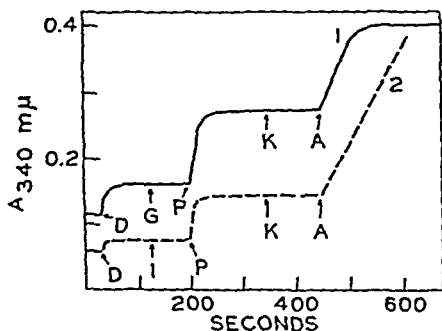
FIG. 2. Reaction sequence in the phosphorylation of ADP coupled to KG oxidation. The reaction components were KG (5 μmoles), DPN (2 μmoles), MgCl$_2$ (10 μmoles), phosphate buffer at pH 7.5 (100 μmoles), CoA (0.05 to 0.12 mg.), and cysteine (10 μmoles) in 3.0 ml. The additions at the appropriate times are indicated by labeled arrows. D refers to KG dehydrogenase (0.5 mg.), G to GDP (0.06 μmole), I to IDP (0.035 μmole), P to GDP phosphorylation enzyme (0.1 mg.), K to NDP kinase (0.05 mg.), and A to ADP (0.07 and 0.5 μmole in Curves 1 and 2, respectively).

1. The DPN reduction that follows the addition of KG dehydrogenase is explained by Equation 1 (1, 2). When the GDP phosphorylation enzyme and GDP are added to the system, a second increase in DPNH, equivalent to the added GDP, is observed. Apparently CoA is released in this step from succinyl CoA (Equation 4), which then participates in KG oxidation to yield DPNH. Since there is no reaction at this stage if GDP is left out, it may be concluded that succinyl phosphate is not an intermediate. A third increase in DPNH is produced when NDP kinase and ADP are added to the system, the DPNH produced at this step being equivalent to the ADP (Equation 5). From previous experiments it is known that ADP is converted stoichiometrically to ATP and an equivalent amount of CoA is released for renewed participation in Equation 1. The phosphorylation enzyme and the kinase cannot be interchanged in this sequence of reations, nor can ADP replace GDP in the second reaction (Equation 4).

The stoichiometry of the reaction between succinyl CoA and GDP which results in CoA release is presented in Table III. The succinyl CoA disappearance is equivalent to the CoASH liberated as required by the formulation in Equation 4. Phosphate, Mg^{++}, and GDP are essential for the breakdown of succinyl CoA catalyzed by the phosphorylation enzyme. If phosphoryl CoA accumulated as an intermediate in the reaction, a decrease in succinyl CoA (hydroxamic acid) would be expected even without the addition of GDP.

For the isolation of the product formed from GDP, the phosphorylation reaction was coupled with the KG dehydrogenase system in order to obtain quantitative conversion. This experiment was carried out in the presence of P^{32}-labeled phosphate. The control consisted of an incubation

TABLE III

Stoichiometry and Component Study of GDP Phosphorylation Reaction

System	$+\Delta$ —SH	$-\Delta$ hydroxamic acid
	$\mu mole$	$\mu mole$
Complete......................	0.21	0.20
No phosphate.................	0.03	0.04
" GDP.......................	0.04	0.04
" enzyme....................	0.03	0.04

The reaction mixture contained succinyl CoA (0.48 μmole), GDP (0.58 μmole), $MgCl_2$ (1.5 μmoles), KBH_4 (1 μmole), phosphate (5 μmoles), GDP phosphorylation enzyme (24 γ), and Tris (12 μmoles) in 0.4 ml. at pH 7.5. The mixture was incubated for 10 minutes at 38°, and aliquots were analyzed for unchanged succinyl CoA (hydroxamic acid reaction) and CoASH formed (nitroprusside).

mixture identical in all respects except that CoA was omitted. The complete system contained KG (15 μmoles), DPN (12 μmoles), CoA (0.12 μmole), cysteine (8 μmoles), $MgCl_2$ (40 μmoles), GDP (2.2 μmoles), phosphate (36 μmoles) with 9.1×10^4 c.p.m. per μmole, KG dehydrogenase (0.5 mg.), and GDP phosphorylation enzyme (0.22 mg.) in 12 ml. at pH 7.5. The mixture was incubated at 25° for 16 minutes. At the end of the reaction, which was followed by observing DPN reduction, the pH of the solution was adjusted to 3.0 to inactivate the enzymes, and 200 μmoles of NaH_2PO_4 were added to dilute the specific radioactivity of P_i. The solution was again adjusted to pH 3.0 and passed through columns of charcoal (Nuchar C-190, 0.9 cm. in diameter and 5 cm. high). The charcoal was washed with cold 0.01 M phosphate at pH 3.0, then with water, and finally the nucleotides were eluted by washing with cold 5 per cent aqueous pyridine. The eluate was brought to pH 7.5 and lyophilized. The residue was dissolved in 0.5 ml. of water, and the nucleotides were

precipitated by the addition of 0.02 ml. of 1 M barium acetate and 0.5 ml. of 95 per cent ethanol. The precipitate was recovered, washed with 50 per cent ethanol, and decomposed with Dowex 50 (H+). The solution of nucleotides was applied as streaks on paper and chromatographed (13). In the control chromatogram two streaks were observed under ultraviolet illumination (R_F 0.24 and 0.50; GDP and DPN, respectively), neither of which had any radioactivity. In the complete system in addition to these two inactive zones a third zone with high P^{32} activity was observed (R_F 0.13). The compound was eluted by exhaustive washing with water, and the eluate was treated with Dowex 50 (H+) to remove ammonium ions and lyophilized. The product had an absorption spectrum identical with that of guanosine at pH 2 and 12. The base obtained after hydrolysis with 1 N H_2SO_4 for 1 hour was identified (15) in two paper chromatographic systems (16, 17). The analytical data appear in Table IV. The P^{32} activity

<div align="center">TABLE IV</div>

Composition of Guanosine Derivative Formed in Phosphorylation Reaction

	μmoles per ml.	Molar ratio
Ribose	1.50	1.00
Guanosine . .	1.55	1.03
Acid-labile P	2.86	1.91
P^{32} activity (1.4 × 10^5 c.p.m.)	1.50	1.00

corresponds to incorporation of 1 mole of phosphate per mole of product. This and the formation of an acid-labile phosphate strongly indicate that the product is GTP.

The synthesis of ATP from the above reaction product (Equation 5) is catalyzed by the second enzyme (NDP kinase). In order to demonstrate this synthesis, 0.7 μmole of the labeled product (specific radioactivity 9.6 × 10^4 c.p.m. per μmole) was incubated for 5 minutes at 30° with $MgCl_2$ (2 μmoles), Tris (5 μmoles, pH 7.2), ADP (0.9 μmole), and NDP kinase (0.1 mg.) in a volume of 0.4 ml. The reaction was stopped by heating for 30 seconds at 75°, and the solution was applied directly to paper for chromatographic separation as described previously. The control, to which no enzyme was added, showed two spots (R_F 0.13 and 0.36), while the complete system showed five spots (R_F 0.12, 0.24, 0.30, 0.36, 0.51). In order to facilitate identification of the spots, reference compounds were applied on the same sheet. The spot with R_F 0.30, which had radioactivity, was eluted and identified as ATP by spectral analysis and by measuring triphosphopyridine nucleotide reduction in the combined hexokinase and glucose-6-phosphate dehydrogenase systems. The specific radioactivity

of the ATP, corrected for decay, was 7.4×10^4 c.p.m. per μmole of adenosine, or approximately 77 per cent of the specific activity of the GTP. The lower specific activity is presumably due to dilution by inert ATP produced from ADP by the action of contaminating myokinase (18). In confirmation, the presence of myokinase in the NDP kinase preparation used in the above experiment was demonstrated by coupling with adenylic deaminase (19). The isolation of GTP from the reaction mixture of the phosphorylation step and of ATP from that of the transphosphorylation step provides additional evidence in support of the two reactions formulated in Equations 4 and 5.

The following nucleotides do not replace GDP in the primary phosphorylation reaction (Equation 4): the 2'-, 3'-, 5'-monophosphates and the triphosphates of adenosine, uridine, guanosine, and cytidine, I-5'-P, ADP, CDP, and UDP. However, IDP can substitute for GDP in the primary phosphorylation as well as in the transphosphorylation with ADP (Fig. 2, Curve 2). As in the case of GDP, the DPN reduced when coupled to the KG dehydrogenase system is equivalent to the IDP added, which indicates stoichiometric conversion to ITP. The reactions involved can be represented by Equations 6 and 7. Transphosphorylation between ITP and ADP has been demonstrated previously (7, 13).

$$\text{Succinyl CoA} + \text{IDP} + \text{P}_i \rightleftharpoons \text{succinate} + \text{CoA} + \text{ITP} \qquad (6)$$

$$\text{ITP} + \text{ADP} \rightleftharpoons \text{IDP} + \text{ATP} \qquad (7)$$
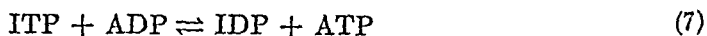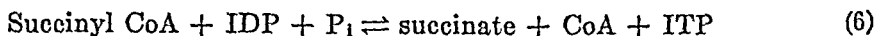
The ratio of the rates of phosphorylation of GDP and IDP was constant at the different stages of purification of the GDP phosphorylation enzyme from pig kidney under identical assay conditions (Table V). The phosphorylation system prepared from pig heart by entirely different procedures (2) showed essentially the same ratio. The reaction rate with IDP was about 45 per cent of the rate obtained with GDP. If the same enzyme catalyzes the phosphorylation of GDP and IDP, as seems likely from the data, the oxygen on carbon 6 of the two purines would be implicated as one of the sites for binding with the enzyme.

According to the formulations in Equations 4 and 6, it should be possible to synthesize succinyl CoA from succinate, CoASH, and GTP or ITP, with only the GDP phosphorylation enzyme. This is indeed the case, as seen in Table VI, where succinyl CoA synthesis has been measured in terms of CoA disappearance. It is interesting to note that the limiting component, which is GTP in one experiment and CoASH in the other, is completely utilized. Thus, with succinate in excess, the reaction can be used for measuring GTP or ITP as well as CoASH. This method of CoA analysis yields results which agree closely with those obtained in a similar stoichiometric assay with the fatty acid activation system (20). In either system

the assay is applicable only to highly purified samples of CoASH. Absence of reaction with only ITP or GTP and CoA (no succinate) indicates that phosphoryl CoA is not accumulated in detectable amounts.

TABLE V

Relative Rates of Phosphorylation of GDP and IDP

Phosphorylation enzyme		Δ 4 per min		$\dfrac{IDP}{GDP}$
Specific activity	Amount used	GDP	IDP	
	γ			
0 70	86	0 060	0.030	0.50
1 54	39	0.060	0 036	0 60
2 20	27	0.066	0 030	0 45
2 53	24	0.066	0.030	0 45
0.30	360	0 108	0 048	0.44

The reaction rates were obtained under standard assay conditions for the phosphorylation enzyme (see the text). IDP was substituted for GDP for the data in the fourth column. The same batch of pig kidney enzyme, at different stages of purification, was used for first four lines. The data in the last line were obtained with a preparation from pig heart (2).

TABLE VI

Succinyl CoA Synthesis with GTP and ITP

	Δ —SH	
	GTP	ITP
	μmole	μmole
Complete system	−0.05	−0.12
No ITP .		0.00
" succinate	0 00	+0.01
" enzyme .	0 00	0.00

The complete GTP system consisted of CoASH (0 24 μmole), GTP (0 043 μmole), succinate (15 μmoles), MgCl₂ (1 μmole), KBH₄ (1 μmole), GDP phosphorylation enzyme (30 γ), and Tris (5 μmoles) in 0 3 ml. at pH 7.2. The incubation time was 15 minutes at 30°. For the ITP system the components were CoASH (0.12 μmole), ITP (0 7 μmole), succinate (5 μmoles), and the others as above. The mixture was incubated for 5 minutes at 30°. At the end of the incubation, saturated NaCl and NaCO₃-NaCN solutions were added directly to the tubes, followed by nitroprusside solution. 0 1 μmole of —SH in 1 ml (1 cm. light path) gave an absorbancy of 0 630 at 540 mμ

Arsenolysis

The arsenolysis of succinyl CoA, catalyzed by the GDP phosphorylation enzyme, can be demonstrated spectrophotometrically by coupling with the

KG dehydrogenase system (3). The arsenolysis is inhibited strongly by phosphate (Fig. 3). The reaction requires Mg^{++}, but is independent of
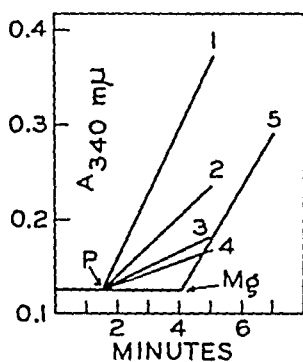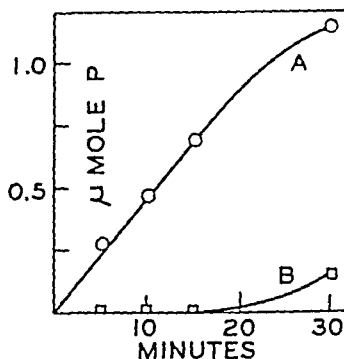
FIG. 3   FIG. 4

FIG. 3. Spectrophotometric demonstration of arsenolysis of succinyl CoA. The reaction mixture consisted of KG, DPN, CoA, $MgCl_2$, and cysteine, as in Fig. 2. The buffer was Tris (30 μmoles, pH 7.5) instead of phosphate, and arsenate (30 μmoles) was also present. The phosphorylation enzyme (1.0 unit) was added at 1.5 minutes (indicated by P). No phosphate was present for Curve 1. Curves 2 to 4 were obtained with 3, 10, and 30 μmoles, respectively, of phosphate in addition to arsenate. For Curve 5, Mg^{++} was left out initially and added at 4 minutes.

FIG. 4. Arsenolysis of ITP. The components were ITP (3 μmoles), $MgCl_2$ (10 μmoles), K arsenate (10 μmoles), cysteine (10 μmoles), Tris (30 μmoles), and phosphorylation enzyme (10 units) in 1.0 ml. at pH 7.5. In addition, for Curve A the mixture contained 0.4 μmole of CoA; for Curve B, 10 μmoles of succinate. Aliquots were taken at 5, 10, 15, and 30 minutes, acidified with trichloroacetic acid, and then assayed for phosphate.

TABLE VII

Stoichiometry of Arsenolysis

Incubation time	$-\Delta$ succinyl CoA		$+\Delta$ $-$SH	
	Arsenate	No arsenate	Arsenate	No arsenate
min.				
5	0.53	0.10	0.80	0.19
15	1.87	0.24	2.02	0.34
25	2.52	0.40	2.65	0.50

The reaction mixture contained succinyl CoA (3.9 μmoles), $MgCl_2$ (5 μmoles), KBH_4 (2 μmoles), Tris (18 μmoles), GDP phosphorylation enzyme (1.5 units), and arsenate (15 μmoles) in 1.5 ml. at pH 7.5. Incubation at 20° under nitrogen.

GDP. The direct evidence for the arsenolysis, presented in Table VII, is consistent with the formulation in Equation 8. The rate of arsenolysis was approximately one-fifth of the rate of the reaction with phosphate and GDP.

$$\text{Succinyl CoA} \xrightarrow{\text{AsO}_4^-} \text{succinate} + \text{CoA} \tag{8}$$
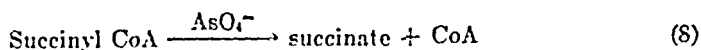
The preparation of phosphorylation enzyme catalyzes also the arsenolytic breakdown of ITP (Equation 9). The reaction requires the addition of CoA and Mg^{++} but not of succinate (Fig. 4). These results have been

$$\text{ITP} \xrightarrow[\text{CoA}]{\text{AsO}_4^-} \text{IDP} + \text{P}_i \tag{9}$$
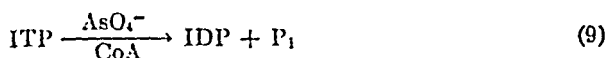
confirmed by qualitative observations on the disappearance of ITP (paper chromatographic method) in the presence of enzyme, CoA, and arsenate. Again, under these experimental conditions, succinate did not replace CoA. It may be noted that the rate of arsenolysis of ITP is considerably slower than that of the arsenolysis of succinyl CoA. These experiments were performed with ITP, since it was more readily available than GTP, and need to be repeated with more highly purified enzymes.

<div align="center">DISCUSSION</div>

The arsenolysis experiments leave no doubt that a phosphorylated compound is formed as an intermediate in the phosphorylation reaction. Green (21) has proposed that phosphoryl S-CoA is the intermediate. Lynen et al. (22) had earlier considered this compound as a possible intermediate in acetyl CoA synthesis. So far there is no definite evidence that such a compound does exist. Our experiments show that *stoichiometric* amounts of this phosphoryl CoA are not formed when either succinyl CoA and phosphate or GTP and CoA are allowed to react (Tables III and VI, respectively).

On the basis of isotope incorporation experiments carried out with a phosphorylation enzyme from spinach, a mechanism involving enzyme-bound phosphoryl CoA as an intermediate has been proposed (23). The mechanism does not explain the succinate-independent arsenolysis of ITP, unless it is assumed that the intermediate dissociates to form free P$_i$ (in the absence of succinate). A detailed analysis of the reaction with more highly purified preparations is necessary before an acceptable mechanism can be suggested for the mammalian enzyme.

<div align="center">*Materials and Methods*</div>

The adenine nucleotides, supplied by the Pabst Laboratories or the Sigma Chemical Company, were rechromatographed on Dowex 1 (Cl$^-$) 2 per cent cross-linked resin (24). The concentration of the chloride ion in the eluate was increased gradually (in steps of 0.01 or 0.02 M). The washing at each concentration was continued until the absorption at 260 mμ of the eluate had dropped to a low or to a constant value over several column

volumes.   Certain critical samples were further purified by paper chromatography (13).   In this separation, all of the adenine nucleotides migrate much faster than the di- and triphosphates of guanosine, inosine, and uridine.   IDP and ITP were prepared from ADP and ATP, respectively, by chemical deamination (25) or were purchased from the Sigma Chemical Company.   Samples of 2'-, 3'-, and 5'-phosphates of adenosine, guanosine, uridine, and cytidine were generous gifts from Dr. W. E. Cohn.   UTP and CDP, supplied by the Pabst Laboratories, were repurified on Dowex 1 (Cl⁻) as described above.   UDP was prepared from UTP by acid hydrolysis or was purified from a sample of crude UDP kindly presented by Dr. S. Lipton.   The preparation of GDP and GTP is described elsewhere (15).   The nucleotide concentration is calculated from the absorption at 260 mμ according to the tables of Volkin and Cohn (26).

CoA was prepared from yeast as described by Beinert et al. (14).   For certain critical experiments it was necessary to repurify the samples by a second Cu precipitation (14) in order to remove traces of other nucleotides. CoA (100 mg.) and glutathione (300 mg.) were dissolved in 15 ml. of water, and the pH of the solution was adjusted to 7.   The solution was allowed to stand at room temperature for 15 minutes, and then 0.75 ml. of 10 N H_2SO_4 was added.   A suspension of 100 mg. of Cu_2O in water was added slowly and with constant stirring.   When the red color disappeared completely, the mercaptide was collected by centrifugation and washed once with 0.05 N H_2SO_4 and then with distilled water until the washings were free of sulfate.   The precipitate was suspended in water, and H_2S was passed through it for several hours.   The Cu_2S was removed, and the supernatant solution was rendered free of H_2S by bubbling nitrogen through it.   The solution (30 ml.) was treated with sodium amalgam (14) and then passed through a column of Dowex 50 (H^+) (15 ml. in volume).   The filtrate and washings were lyophilized.   The yield was 41 mg., and the purity of the CoA was 82 per cent.

Succinyl CoA was synthesized essentially by the method of Simon and Shemin (27), except that a 3- to 4-fold excess of succinic anhydride in dioxane was used.   When the reaction was completed, as shown by no further decline in —SH (nitroprusside reaction), the nucleotides were precipitated by the addition of excess barium acetate and alcohol.   The precipitate was washed with alcohol and dried.   The Ba^{++} was removed with Dowex 50 (H^+), and the solution was then neutralized and lyophilized. The succinyl CoA in these preparations amounted to 0.7 to 0.8 μmole per mg.   The preparation was stable for 12 months when kept over desiccant at $-10°$.   The sources of other materials have been indicated previously (1).

KG dehydrogenase was prepared as described previously (10) and then

subjected to an isoelectric precipitation. The dialyzed enzyme was diluted with 0.01 M phosphate, pH 7.0, to approximately 5 mg. of protein per ml., and the pH of the cold solution was adjusted to 5.8 to 5.9 with 1 per cent acetic acid. It was immediately centrifuged and the precipitate was dissolved in 0.01 M phosphate, pH 7.5, to give a protein concentration of approximately 20 mg. per ml. The solution was clarified, if necessary, by recentrifugation.[4]

Determination of Phosphate in Presence of Arsenate—The reaction mixture was deproteinized with 5 per cent trichloroacetic acid. To an aliquot of the extract containing 0.1 to 1.0 μmole of phosphate and less than 10 μmoles of arsenate in 0.2 to 0.5 ml., 10 to 50 mg. of repurified sodium dithionite ($Na_2S_2O_4$) were added. The mixture was allowed to stand at room temperature for about 5 minutes and then diluted to the required volume for phosphate analysis (29). Under these conditions, the arsenate is reduced to arsenite and no color is produced in the reaction. The dithionite was rigorously purified by the method of Hill (30) and recrystallized twice. Purified samples can be stored for some months *in vacuo* over P_2O_5. Impure preparations produce a white precipitate during phosphate analysis or even after adding it to the trichloroacetic acid extract.

The nitroprusside reaction was carried out by a modification of the method described by Grunert and Phillips (31). Other methods used have been described previously (1).

SUMMARY

The phosphorylation of ADP coupled to the breakdown of succinyl CoA in animal tissues is a two-step reaction involving two different enzymes and GDP. The product of the first reaction catalyzed by the GDP phosphorylation enzyme is GTP. The second step, transphosphorylation from GTP to ADP, is catalyzed by NDP kinase. GTP has been isolated from the reaction and identified. For the synthesis of succinyl CoA from ATP both enzymes and GDP are necessary. However, only the phosphorylation enzyme is necessary for succinyl CoA synthesis when GTP is used instead of ATP. IDP can substitute for GDP in the system but less effectively.

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[4] The DPNH oxidizing activity (28) of such a preparation was only 10 to 20 per cent of that observed by Huennekens *et al.* Before this step the activity was 2- to 3-fold higher and in agreement with our earlier calculations that the "diaphorase" contamination amounted to 1 to 2 per cent of the total protein (10). It may be appropriate to emphasize that Huennekens *et al.* have compared the DPNH oxidizing and KG dehydrogenase (DPN reduction) activities of the enzyme preparation at two different pH values, the former at pH 10 and the latter at pH 7.4. The observed activity, if assumed to be due to "diaphorase," would amount to less than 1 per cent of the total protein in the preparation.

The GDP phosphorylation enzyme catalyzes the arsenolytic breakdown of succinyl CoA. The reaction is inhibited by phosphate. For the arsenolysis of ITP, CoA, but no succinate, is necessary.

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ISOLATION OF GUANOSINE DI- AND TRIPHOSPHATES FROM YEAST*

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The enzyme system from animal tissues that couples the breakdown of succinyl CoA to the phosphorylation of ADP has been separated into two fractions.[1] An additional nucleotide cofactor was found to be necessary when highly purified preparations of CoA and ADP were used in the reaction (1, 2). It was also shown that this nucleotide is present in yeast extracts. A method for the isolation of the nucleotide and its identification as guanosine-5′-pyrophosphate or GDP are described in this paper.

Results

Assay—The assay of the nucleotide is carried out spectrophotometrically in a coupled system, as outlined in the preceding paper (2). The nucleotide is quantitatively phosphorylated, and an equivalent amount of DPN is reduced according to Equations 1 and 2. Applications of the

$$\text{KG} + \text{DPN}^- + \text{CoA} \rightarrow \text{succinyl CoA} + \text{DPNH} + \text{H}^- + \text{CO}_2 \qquad (1)$$

$$\text{Succinyl CoA} + \text{GDP} + \text{P}_i \rightleftharpoons \text{succinate} + \text{CoA} + \text{GTP} \qquad (2)$$

assay at different stages of purification and the conditions for the assay

[1] The following abbreviations are used: G-2′-P, G-3′-P, G-5′-P, GDP, and GTP for the 2′-, 3′-, 5′-, di-, and triphosphates of guanosine. Similar symbols are used for the nucleotides of adenosine (A), uridine (U), and inosine (I). KG is used for α-ketoglutarate, KGD for KG dehydrogenase, DPN or DPN$^+$ for diphosphopyridine nucleotide, DPNH for reduced DPN, CoA for coenzyme A, P_i for inorganic phosphate, Tris for tris(hydroxymethyl)aminomethane, and A_{260} for absorbancy or log I_0/I at 260 mμ.

are shown in Fig. 1. With purified samples of GDP (*e.g.*, Curve 3) the reaction terminates when the added GDP is consumed. With less pure preparations, the assay is complicated by the continued DPN reduction. Correction for this can be applied readily by extrapolation of the rate back to the time of addition of the phosphorylation enzyme, as shown in Fig. 1. The corrected value for the DPNH produced is equal to GDP concentration in the sample. The continued DPN reduction is caused by two factors. First, the GDP phosphorylation enzyme contains small
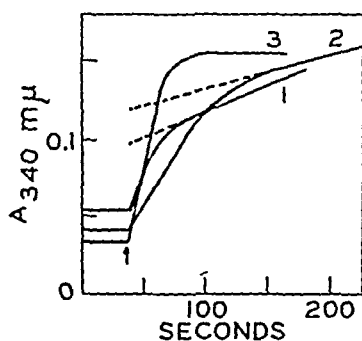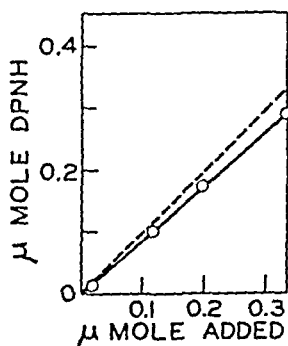
FIG. 1   FIG. 2

FIG. 1. Assay of GDP at different stages of purification. The assay system contained KG (5 μmoles), DPN (1 μmole), MgCl$_2$ (10 μmoles), phosphate (100 μmoles) at pH 7.5, cysteine (20 μmoles), CoA (0.02 μmole), sample for assay, and KGD (0.05 mg.) in 3.0 ml. The sample for assay, CoA, and cysteine were incubated for 3 to 5 minutes at 30° at pH 7.5 before mixing with the other components. The reaction with GDP was initiated by the addition of the phosphorylation enzyme (0.1 to 0.2 unit) at the time indicated by the arrow. Curve 1 was obtained with 0.9 mg. of crude concentrate from the charcoal column, Curve 2 with 0.4 mg. of Fraction 1, and Curve 3 with a sample of eluate from the Dowex 1 column.

FIG. 2. Relation between GDP added and DPNH formed. Assay conditions as in Fig. 1. The GDP concentration (added) was calculated from the A_{260} of the sample. From the data, the sample appeared to be 90 per cent pure. The dotted line shows the expected relationship if the sample were 100 per cent pure.

amounts of enzymes that catalyze transphosphorylation from GTP to other mono- and dinucleotides. Second, crude samples of GDP contain other nucleotides which accept phosphate from GTP. As a result GDP is produced by transphosphorylation which reacts again in the system. By selecting appropriate levels of KGD and the phosphorylation enzyme, it is possible to obtain a good estimate (±10 per cent) of the GDP content of even relatively crude samples.

The DPNH produced is practically equivalent to the GDP added over wide limits with purified samples (Fig. 2). This holds true with crude samples also, except that the correction is more difficult to apply at higher levels of nucleotide. In practice, the level is adjusted to give an absorbancy change of 0.03 to 0.10 (equivalent to 0.015 to 0.050 μmole of GDP).

Early in the investigation, a small sample of active nucleotide was isolated from commercial samples of ADP. Its tentative identification as a guanosine phosphate aided greatly the subsequent purification. The characteristic ratios of absorption at different wave-lengths in the ultraviolet region at acid and alkaline pH (3)[2] have been highly useful in this work. For example, the A_{280}/A_{260} at pH 2 of the 5'-phosphates of guanosine is 0.68, of adenosine 0.22, of uridine 0.38, and of cytidine 2.1. The ratios change to 0.60, 0.15, 0.33, and 0.99, respectively, at pH 12.

Isolation of GDP and GTP

Analysis for GDP in boiled extracts of yeast showed that its concentration was approximately 180 μmoles per kilo of dry yeast.[3] This value is in close agreement with that calculated from the recent chromatographic data of Schmitz (4).

Nucleotide Concentrate—In testing different sources for the active nucleotide, it was found that the crude CoA concentrate prepared from yeast (5) by adsorption on charcoal, followed by elution with aqueous pyridine and precipitation with acetone, was a rich source. Different samples assayed from 20 to 25 μmoles of GDP per gm., corresponding to a yield of 110 to 150 μmoles per kilo of original dry yeast. The method for the preparation of concentrate was modified slightly as follows.[4] Barium acetate solution, 1 M, was added to the cooled pyridine eluate (1.8 ml. per liter), followed by cold 95 per cent ethanol to bring the final ethanol concentration to approximately 50 per cent. The gray precipitate that formed immediately was allowed to settle overnight. The supernatant fluid was siphoned off, and the precipitate was collected by centrifugation. After being washed once with 50 per cent ethanol and twice with acetone, it was dried over H_2SO_4 in a desiccator. The yield of the gray powder was 4.3 to 5.0 gm. and of GDP, 110 to 150 μmoles per kilo of yeast. The CoA recovery was the same as that obtained by the original procedure, which involved concentration of the eluate and precipitation of the nucleotides with acetone (5).

Separation of Guanosine Nucleotides and CoA—The concentrate obtained by precipitation with Ba^{++} was stirred with cold 0.07 N HNO_3 (2 liters per 100 gm.), and 3 N HNO_3 was added dropwise to maintain the pH at 2. The solution was kept cold throughout the fractionation except where specified. The slurry was centrifuged for 15 to 30 minutes at 2000 $\times$ g,

[2] We are deeply indebted to Dr. W. E. Cohn for supplying the data before publication as well as for gifts of 2'-, 3'-, and 5'-phosphates of adenosine, guanosine, cytidine, and uridine. He also suggested that the cofactor may be a guanosine derivative from its spectrum.

[3] Primary dried yeast, strain G, Anheuser-Busch, Inc., St. Louis, Missouri.

[4] In the original procedure (5) Nuchar C-190 was used. We have found that Nuchar C is just as satisfactory as Nuchar C-190.

and the residue was again stirred with the same volume of 0.02 N HNO₃. A small amount of powder, mostly charcoal, remained insoluble and was discarded after centrifugation. The supernatant solutions were combined and adjusted to pH 6.6 with 2 N NaOH, allowed to stand in the refrigerator for 30 minutes or more, and then centrifuged as before. The residue (Fraction 1) was washed three times with water at room temperature (600 ml. each time per 100 gm. of original concentrate). The washings were combined with the supernatant solution, and cold 95 per cent ethanol was added to a final concentration of 50 per cent. After 30 minutes, the precipitate was recovered by centrifugation, washed once with 50 per cent EtOH, twice with acetone, and dried (Fraction 2).

Fraction 1 contained 15 to 20 per cent of the original material (dry weight) and over 70 per cent of the GDP. The GDP concentration at this stage was usually 80 to 110 μmoles per gm. of dry weight. Fraction 2 had 30 to 40 per cent of the original solids and over 80 per cent of the CoA. For the recovery of CoA, Fraction 2 was suspended in water and ground with 2- to 3-fold excess of Dowex 50 (H⁺) until the precipitate dissolved. The Dowex 50 was washed with water, and the combined extract and washings were diluted to 800 ml. per 100 gm. of original nucleotide concentrate. Subsequent operations involving precipitation with cuprous oxide in the presence of excess glutathione were carried out as described previously (5). The yield of CoA by this modification was lower (about 50 to 70 per cent of the reported yield per kilo of dry yeast), and the purity varied from 50 to 60 per cent. No attempts have been made to improve the yield or purity of CoA.

Chromatography on Dowex 1—Fraction 1 was rendered free of Ba⁺⁺ by mixing with Dowex 50 (H⁺) and water and stirring the suspension until the precipitate disappeared. The Dowex 50 was removed, the pH of the extract was adjusted to about 7, and the nucleotides were chromatographed on a Dowex 1, chloride, column. In our experience, the 2 per cent cross-linked resin (X2) gave better resolution than the X8 resin. Approximately 60 μmoles of nucleotides, calculated from A_{260} with the ε of adenosine, were used per ml. of wet packed resin. A description of a typical run is given below, and a flow sheet of the isolation is presented in Diagram 1.

A sample of Fraction 1 (4.5 gm. containing 4.7 mmoles of adenosine equivalent, calculated from A_{260}, and 520 μmoles of GDP) was decomposed with Dowex 50 (H⁺). The neutralized solution (400 ml.) was passed through a column of Dowex 1 (Cl⁻) (X2, 200 to 400 mesh, 2.5 cm. in diameter and 18 cm. in height). The flow rate through the column was maintained at 4 ml. per minute by applying a pressure of 1 to 2 pounds per sq. in. from a nitrogen tank.[5] The column was washed with 300 ml. of

[5] A pressure reducing valve, No. 70 regulator from the Matheson Company, Inc., East Rutherford, New Jersey, was used with the tank.

water and then with 2 to 3 liters of 0.01 N HCl containing 0.01 N NaCl until the A_{260} of the eluate dropped to a low value and remained reasonably constant. Approximately 40 to 60 per cent of the material, in terms of A_{260}, was eluted from the column at this stage. This part of the procedure was conveniently carried out overnight, and the eluate was collected in one large fraction. The A_{280}/A_{260} ratio of this fraction indicated that ad-

4.7 kilos dry yeast (850 μmoles GDP)

Extract with aqueous acetone
Adsorb on charcoal
Elute with aqueous pyridine
Ppt. with Ba acetate

Ba nucleotide concentrate
25 gm., 660 μmoles GDP

Extract with HNO₃

Adjust pH to 6.6

Wash; dry
Fraction 1, 4.5 gm., 520 μmoles GDP   Add EtOH

Dowex 1, Cl⁻   Wash; remove Ba⁺⁺
Fraction 2

Cu₂O precipitation

GDP, 440 μmoles
GTP, 60 μmoles   CoA
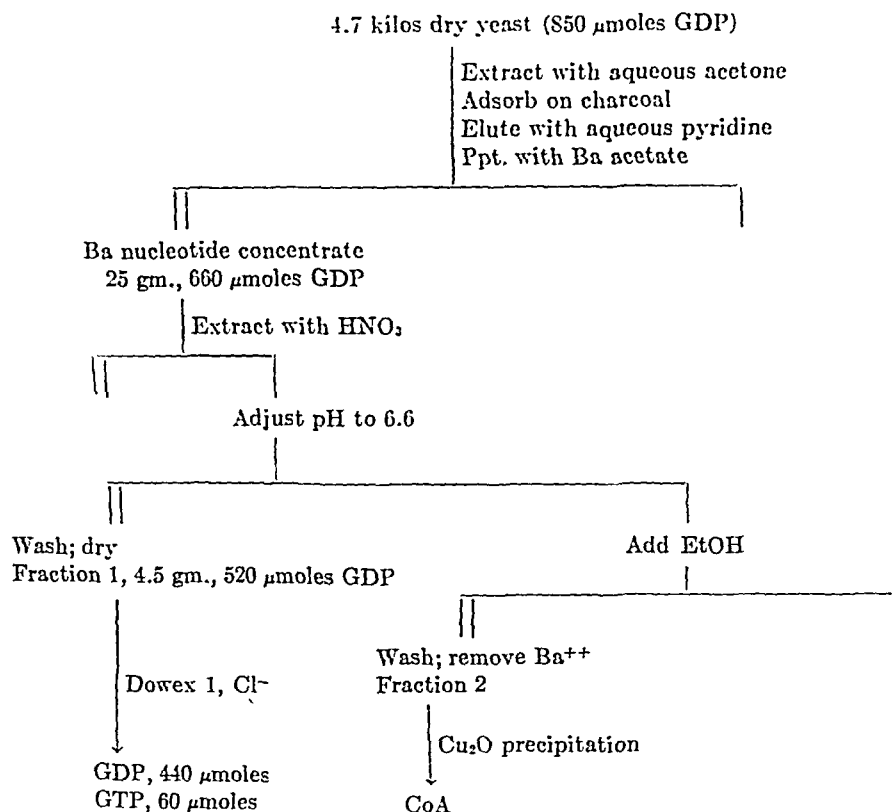
DIAGRAM 1. Flow sheet for the isolation of GDP, GTP, and CoA from yeast

enine nucleotides were the major components. When the A_{280}/A_{260} of the eluate rose to over 0.5, the concentration of NaCl in the eluent was increased to 0.04 N, the HCl concentration being the same (0.01 N). The eluate was collected in 200 ml. fractions and immediately adjusted to pH 6.5 to 7.0. GDP and GTP appear to be more labile than the corresponding adenine nucleotides, and greater care is necessary to prevent hydrolysis. When most of the GDP was eluted, the NaCl concentration was increased to 0.08 N, which resulted in elution of ATP from the column. The elution pattern and the A_{280}/A_{260} of the fractions are shown in Fig. 3. The elution was continued until GTP was recovered. UDP

and UTP follow GDP and GTP on the column and may be recovered by a more gradual increase in NaCl concentration.

Fractions of GDP containing over 0.3 μmole per ml. were pooled and concentrated by lyophilizing so that the final Cl^- concentration was less than 1 M. The solution was cooled in ice, and 1 M $BaCl_2$ was added in a 3-fold excess over the nucleotide concentration. GDP was precipitated as the barium salt by the addition of cold 95 per cent ethanol to a final concentration of about 50 per cent. The mixture was allowed to stand overnight in the refrigerator and the precipitate recovered by centrifugation. It was washed twice with small volumes (5 to 10 ml.) of cold water to remove any precipitated NaCl, then with alcohol and ether, and dried.
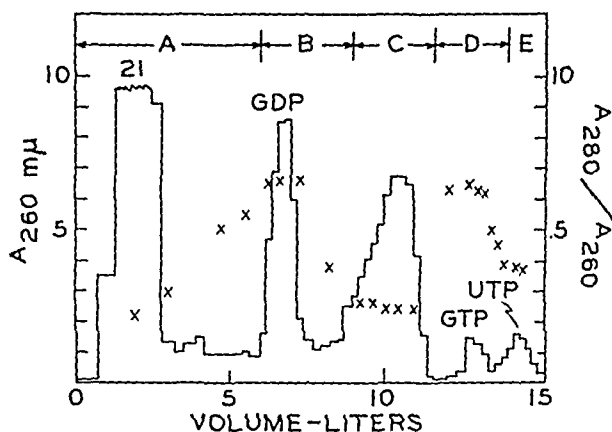
FIG. 3. Elution pattern of chromatogram. The eluents were (A) 0.01 N HCl, 0.01 N NaCl; (B) 0.01 N HCl, 0.04 N NaCl; (C) 0.01 N HCl, 0.08 N NaCl; (D) 0.01 N HCl, 0.12 N NaCl; (E) 0.01 N HCl, 0.13 N NaCl. The A_{260} of the main fraction was 21.0. $\times$ represents the A_{280}/A_{260} of the fraction.

The same procedure was followed to obtain GTP from the eluate. The yield of the GDP fraction (Ba salt) was 0.35 gm. and that of the GTP fraction 0.04 gm. The nucleotides were stored as the barium salts over desiccant at $-10°$. The GDP recovered was 440 μmoles by enzymatic analysis and 490 μmoles by A_{260}. The yield of GTP was 56 μmoles by A_{260}. This value was in close agreement with the enzymatic assay described subsequently. On a paper chromatogram, with the isobutyric acid-ammonium isobutyrate solvent (6), the GDP appeared as two spots; the faster moving component appeared as an extremely faint spot. These results were confirmed by rechromatography on Dowex 1 (Cl⁻) as well as on Dowex 1 (formate) as described by Hurlbert et al. (7).[6] The minor component, never more than 10 per cent, was identified as G-5′-P by comparison with a reference sample as well as by absorption spectrum. Pre

[6] We are indebted to Dr. Van R. Potter for making available, before publication, this method for separation of nucleotides on Dowex 1 (formate).

sumably it arose by breakdown during the procedures subsequent to Dowex chromatography. No contamination by other (non-guanine) nucleotides has been observed so far in several preparations obtained as described above.

Identification of GDP

All of the analytical data reported here were obtained on the same sample. The free nucleotide was prepared by suspending the barium salt in water and stirring with Dowex 50 (H$^+$) until the white precipitate dissolved. The solution was lyophilized.
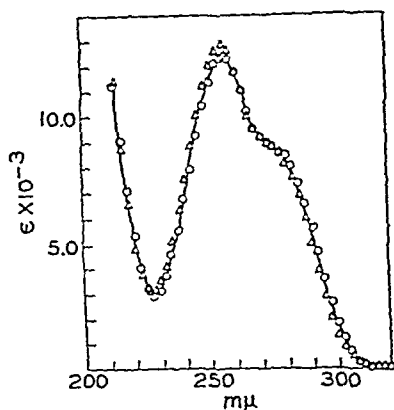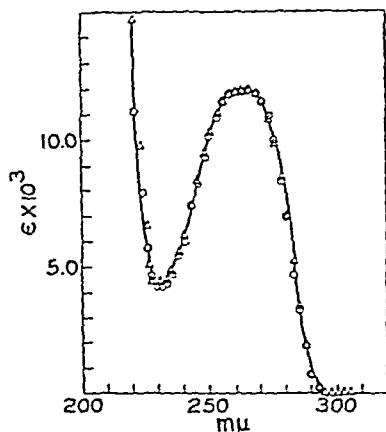
FIG. 4   FIG. 5

FIG. 4. Absorption spectra at pH 2. The solid line refers to the active nucleotide, △ to guanosine, and ○ to G-5'-P. The $\epsilon_{260\ m\mu}$ (molar extinction coefficient) of guanosine phosphates was taken to be 11.8 × 10^3 (3).

FIG. 5. Absorption spectra at pH 12. Symbols as in Fig. 4.

Spectrum—The absorption spectra of the sample in 0.01 N HCl and in 0.01 N NaOH are shown in Figs. 4 and 5. The spectra were identical with the spectra of guanosine and G-5'-P under similar conditions. In alkaline solution there was a characteristic shift and a general broadening of the absorption peak. After hydrolysis in 2 N HCl at 100° for 2 hours, the spectrum was identical with that of guanine.

Base—The acid hydrolysate was chromatographed on paper with acidic and basic solvents: 5 per cent ammonium citrate at pH 3.6-isoamyl alcohol (8) and butanol-diethylene glycol-water in NH$_3$ vapor (9). In both systems the R_F value of the base derived from the cofactor was identical with the R_F of authentic guanine (0.51 and 0.26 in the acidic and alkaline systems, respectively). When the sample and guanine were combined, a single spot appeared without any trace of separation.

Ribose—Ribose was identified and estimated by the orcinol reaction (10). The absorption spectrum of the complex was identical with that obtained with A-5'-P. The rate of color development was also identical with that of a sample of A-5'-P.

Phosphate—The nucleotide contained acid-labile phosphate (1 N H_2SO_4, 100°, 10 minutes), which was estimated by the method of Fiske and Subbarow (11). For total phosphate, the sample was digested as described by King (12).

The results of the chemical analyses are presented in Table I. The purity of the preparation, calculated from A_{260}, was 86 per cent. Preparations, with a purity of 91 per cent, have also been obtained. There was no loss in weight on drying *in vacuo* over P_2O_5 at 56° for 2 hours.

TABLE I

Chemical Analysis of Guanosine Nucleotides from Yeast

	Diphosphate		Triphosphate	
	μmoles per mg.	Ratio	μmoles per ml.	Ratio
Guanosine (A_{260})...... .	1.94	1.00	3.84	1.00
Ribose (orcinol) . . .	1.81	0.94		
Phosphate (total) .	3.59	1.85	11.9	3.10
" (acid-labile)	1.74	0.90	7.98	2.08

Nucleotide Pyrophosphatase Action—The chemical analysis suggested that the isolated nucleotide may be GDP. Confirmatory evidence of this structure was obtained by the action of nucleotide pyrophosphatase (NPPase) (13).[7] 1 mole of phosphate per mole of guanosine was liberated from the compound (Fig. 6). The rate of hydrolysis was 17 times faster than that of G-5'-P. The other isomeric guanosine monophosphates (2'-, 3'-) were hydrolyzed even more slowly.

The product of the hydrolysis from one of the experiments was chromatographed in the isobutyric acid-ammonium isobutyrate system (6). The R_F values of the reference compounds, which were clearly separated from one another, were as follows: G-2'-P, 0.34; G-3'-P, 0.30; G-5'-P, 0.23. The R_F of the NPPase hydrolysis product was 0.23. A mixed sample of the product and G-5'-P showed no resolution.

Nucleoside Diphosphatase (NDPase) Action[8]—Further evidence was obtained by the use of a phosphatase from pig kidney which liberates one phosphate from GDP, UDP, and IDP, but is inactive towards ADP,

[7] This enzyme was a gift from Dr. T. P. Singer and Dr. E. Kearney.

[8] We are indebted to Dr. Miriam Jacob for carrying out the c in this section.

ATP, UTP, and ITP or the corresponding 5′-monophosphates (14).[9] GDP (159 μmoles) was incubated with the phosphatase (12 mg.) and MgCl₂ (100 μmoles) in 10 ml. of 0.035 M Tris at pH 7.5 for 20 minutes at 38°. The enzyme was then inactivated by heating for 2 minutes at 100°, and the denatured protein was removed. The products were separated
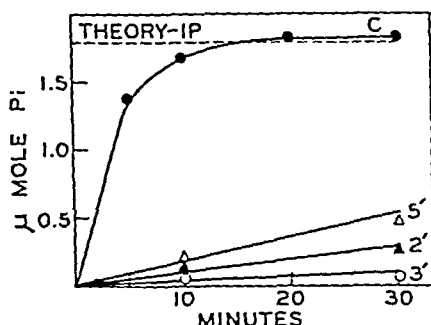
FIG. 6. Action of nucleotide pyrophosphatase on the active nucleotide. The reaction mixture, consisting of 1.8 μmoles of nucleotide, 5 μmoles of Tris, and 0.1 mg. of NPPase in 0.5 ml. volume at pH 7.6 was incubated at 38°. Aliquots were analyzed at intervals for P_i. C refers to nucleotide, and 2′-, 3′-, and 5′- to the three isomeric monophosphates of guanosine.

TABLE II

Action of 5′-Nucleotidase on Product of Hydrolysis of GDP by Nucleoside Diphosphatase

Incubation time	P_i
min.	*μmoles*
15	1.55
30	2.10
60	3.60

The reaction mixture contained glycine, pH 8.5 (100 μmoles), MgCl₂ (10 μmoles), 5′-nucleotidase (0.2 mg.), and guanosine nucleotide (4.0 μmoles) in 1.0 ml. Incubation at 38°.

on Dowex 1 (formate), X2. The column was washed first with 0.5 M formic acid, and G-5′-P was then eluted with 1 M formic acid. The nucleotide was precipitated as the barium salt, the over-all yield being 72 μmoles. Its absorption spectrum was similar to that of guanosine. Total phosphate analysis showed 1 mole of phosphate per mole of base. The R_F was the same as that of G-5′-P. The action of purified 5′-nucleotidase of snake

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[9] Recently, similar phosphatase activity has been demonstrated in other tissues (15–17).

venom (*Crotalus adamanteus*) (18)[10] on this compound was studied. Table II shows that the nucleotidase catalyzed the hydrolysis of the product derived from GDP. The rate of hydrolysis was essentially the same with A-5'-P, while A-2'-P, A-3'-P, G-2'-P, and G-3'-P were not hydrolyzed over the same period of time under similar conditions.

In agreement with the above data on the enzymatic liberation of one phosphate group, the titration curve of the cofactor showed three distinct breaks corresponding to pK values of 2.80, 6.40, and 9.45.[11] The pK of 6.40 indicates that the terminal phosphate group was unesterified.

Entirely independent evidence for the structure of the nucleotide was obtained with a sample of synthetic G-5'-P.[12] This sample did not replace
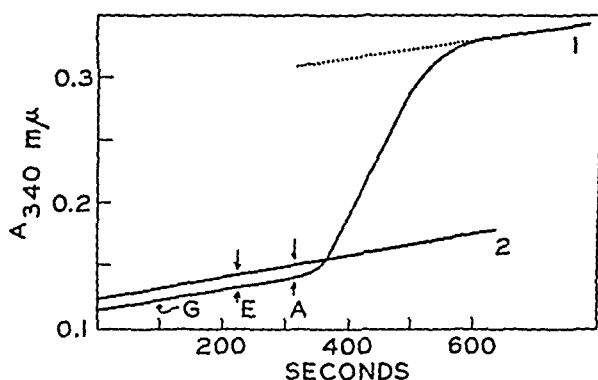
Fig. 7. Synthesis of GDP from G-5'-P. The reaction mixture was similar to that shown under Fig. 1. *G* stands for 0.04 μmole of synthetic G-5'-P, *E* for 0.05 ml. of a mixture of GDP phosphorylation enzyme and NDP kinase, and *A* for 0.2 μmole of ATP. In the experiment represented by Curve 2, G-5'-P was omitted and there was no additional reaction.

the active nucleotide in the primary phosphorylation (Equation 2). However, when ATP was also added to the system, DPN reduction was resumed in the coupled KG oxidation system (Fig. 7). The DPNH produced was 0.085 μmole, which corresponds approximately to twice the G-5'-P added. The first reaction in this sequence is the synthesis of GDP from G-5'-P and ATP by transphosphorylation catalyzed by the nucleoside monophosphate kinase which is present in the GDP phosphorylation enzyme (Equation 3) (15, 19). The GDP reacts in the coupled phos-

$$\text{ATP} + \text{G-5'-P} \rightleftharpoons \text{ADP} + \text{GDP} \qquad (3)$$

phorylation system (Equations 1 and 2), producing 1 equivalent of DPNH.

[10] The purified 5'-nucleotidase was kindly provided by Dr. E. Herbert and Dr. Van R. Potter.

[11] This experiment was kindly performed by Dr. R. M. Bock.

[12] The sample was a gift from Dr. H. G. Khorana and Dr. R. W. Chambers.

The GTP produced in turn phosphorylates the ADP (Equation 4) (2) and itself reverts GDP, giving rise to the 2nd molecule of DPNH.

$$GTP + ADP \rightleftharpoons GDP + ATP \qquad (4)$$
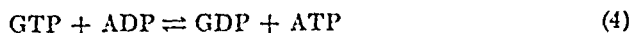
On the basis of the above evidence it is concluded that the nucleotide isolated from yeast was guanosine-5'-pyrophosphate or GDP.

Identification of GTP

The second guanosine nucleotide isolated from the column has been analyzed by essentially the same methods as those described for GDP. The compound contains 3 moles of phosphate, of which 2 are acid-labile, per mole of guanosine (Table I). The identity of guanosine is based on the absorption spectra at pH 2 and 12. The free base produced by prolonged acid hydrolysis was identified by paper chromatography as described previously. Although this guanosine nucleotide does not replace GDP in the phosphorylation reaction (Equation 2) when measured by coupled DPN reduction in the KG dehydrogenase system (Equation 1), it can act as the cofactor for the over-all coupled phosphorylation of ADP (coupling of Equations 1, 2, and 4). The first step in this series of reactions is transphosphorylation with ADP, whereby GDP is liberated (Equation 4) (2).

Furthermore, synthesis of succinyl CoA from succinate and CoA occurs in the presence of this guanosine nucleotide and the GDP phosphorylation enzyme. ATP is not required, nor is the nucleoside diphosphokinase (2) necessary. Data demonstrating this synthesis have been presented previously (2). These observations strongly suggest that the compound is GTP.

DISCUSSION

Guanosine nucleotides have been recognized independently in several laboratories during the analysis of acid-soluble nucleotides from different tissues. Our isolation, on the other hand, was initiated by the recognition of a new cofactor (2). GTP was reported to occur in commercial samples of ATP (20). The presence of guanosine mono-, di-, and triphosphates in animal tissues was clearly demonstrated by Hurlbert et al. (7). Strominger (21) has reported on the occurrence of GDP in the hen oviduct. Besides these compounds, there occurs in yeast another nucleotide, guanosine diphosphomannose (22), the function of which is yet unknown. The GDP isolated by us does not contain any reducing sugar. The liberation of 1 mole of phosphate by the action of NPPase or NDPase, as well as the presence of a secondary phosphate hydroxyl (pK 6.4), excludes mannose from our preparation. Also, the guanosine content of the samples was too high

to be consistent with the molecular weight of GDP-mannose. Furthermore, the active nucleotide (GDP) can be synthesized enzymatically from G-5'-P and ATP by transphosphorylation, which again excludes the presence of mannose.

The barium nucleotide fraction (Fraction 1, Diagram 1) precipitated at pH 6.6 is a good source of many nucleotides. Uridine, cytidine, and thymidine nucleotides have been recognized by their characteristic A_{280}/A_{260} ratios in the eluates from Dowex 1 columns. By selecting the conditions carefully, it should be possible to recover any of these nucleotides from the chromatogram. In one experiment UTP was obtained in approximately the same yield as GTP (Fig. 3).

Materials and Methods

Some of the methods and sources of materials used in this study have been described previously (2). Guanine and guanosine were obtained from the Schwarz Laboratories, Inc.

Paper chromatography (ascending) was carried out on Whatman No. 4 paper, 45 cm. $\times$ 30 cm., in battery jars at room temperature. Reference compounds were chromatographed on the same paper whenever identification of a compound was the aim. Absorption spectra of the nucleotides were measured in the Cary spectrophotometer. For other absorption measurements the Beckman DU or DUR spectrophotometer was used.

KG dehydrogenase, GDP phosphorylation enzyme, and nucleoside diphosphokinase were prepared as described previously (2). Other methods are discussed in the text.

SUMMARY

The cofactor which is necessary for the phosphorylation of ADP coupled to the breakdown of succinyl CoA has been isolated from yeast and identified as GDP. The method of isolation includes recovery of GTP as well as of CoA. The possibility of isolation of a large number of other nucleotides from the Dowex 1 chromatogram is indicated.

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MANNOSE ISOMERASE OF PSEUDOMONAS SACCHAROPHILA*

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In the past few years, several enzymes have been discovered which catalyze the aldose-ketose isomerization of free sugars (1–4). In the course of studies on the metabolism of fructose in *Pseudomonas saccharophila*, a new enzyme which interconverts fructose and mannose has been found. This enzyme, named "mannose isomerase," is of interest not only because it is the first isomerase with a high affinity for free hexoses, but also because it appears almost invariably in mutant strains of *P. saccharophila* which are capable of growing with fructose as substrate (5). The parent strain, which cannot utilize free fructose, does not produce the enzyme when grown with sucrose as carbon source, even when free fructose is added to the medium. The present report deals with the properties of the enzyme obtained from an "F" strain, which is characterized by being able to grow with sucrose or fructose but not with glucose as substrate.

Methods

P. saccharophila, strain F-1, was grown at 30° with constant agitation on a rotary shaker in a medium containing 0.033 M KH_2PO_4-Na_2HPO_4 buffer at pH 6.8, 0.05 per cent magnesium sulfate, 0.1 per cent ammonium chloride, 0.01 per cent ferric ammonium citrate, 0.001 per cent calcium chloride, and 0.25 per cent fructose. The cells were harvested by centrifugation, washed twice with water, and stored as a paste at −20°.

Cell-free extracts were prepared by sonic disintegration of a 20 per cent (weight per volume) suspension of the cells in 0.1 M Tris-HCl[1] or Tris-H_2SO_4 buffer containing 0.01 M glutathione, at pH 7.4, in a Raytheon magnetostrictor of 9 kc. for 20 minutes. The unbroken cells and particulate débris were removed by centrifugation at 22,000 × g for 30 minutes.

Reducing sugars were determined by the method of Schales and Schales (6). Fructose was estimated according to Roe (7). Other ketoses were

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[1] The following abbreviations are used: SAS, ammonium sulfate saturated at 0° and neutralized with NH_4OH; Tris, tris(hydroxymethyl)aminomethane (molarity of Tris buffers expressed in terms of Tris); EDTA, ethylenediaminetetraacetic acid (Versene) adjusted to pH 7.4 with H_2SO_4.

measured with the cysteine-carbazole reaction of Dische and Borenfreund (8). Xylulose was determined after 60 minutes incubation with the cysteine-carbazole reagents by comparison with known standards. (0.01 μmole gave a reading of 17.5 with a No. 54 filter in the Klett colorimeter.) Mannose-6-phosphate was prepared by the phosphorylation of mannose with adenosine triphosphate and yeast hexokinase. The "water-soluble, alcohol-insoluble" barium salts from such preparations contained less than 7 per cent of glucose and fructose esters. Aldoses were determined by the method of Willstätter and Schudel (9). Protein was measured by the method of Weichselbaum (10).

D-Xylulose, sedoheptulose, and fructose uniformly labeled with C^{14} were obtained from Dr. W. Z. Hassid and Dr. E. W. Putman. A sample of o-nitrophenylhydrazone of ribose was kindly supplied by Dr. S. S. Cohen. D-Mannoheptulose and D-glucoheptulose were gifts of Dr. A. C. Neish. D-Rhamnose was kindly synthesized by Dr. C. E. Ballou. All other compounds were commercial preparations.

<div style="text-align:center">EXPERIMENTAL</div>

<div style="text-align:center">Interconversion of Fructose and Mannose</div>

When crude or partially purified enzyme preparations of fructose-grown cells of the F-1 strain of *P. saccharophila* were incubated with fructose, a partial disappearance of ketose as measured by the Roe test was observed. This disappearance was not accompanied by a decrease in reducing sugar either before or after treatment with zinc sulfate and barium hydroxide. The crude enzyme was incubated with fructose-C^{14}, and the reaction mixture was deproteinized by boiling and chromatographed on paper. On chromatograms developed with butanol-acetic acid-water (4:1:5), a single spot, identical with that for fructose and mannose, could be found by treatment with p-anisidine-HCl or by radioautography. On chromatograms developed with phenol, a single new spot in addition to that corresponding to fructose was located by both methods. The R_F of this spot corresponded closely to that of mannose. These observations suggested that mannose and no other compound was produced from fructose under the conditions of the experiment.

Further identification of mannose as the product of the reaction was based on the formation of an insoluble phenylhydrazone of this compound at room temperature. A mixture containing 40 ml. of 20 per cent cell-free preparation, 6 mmoles of fructose, 5 mmoles of $MgCl_2$, and 2 mmoles of Tris buffer, pH 7.4, in a total volume of 72 ml. was incubated for 30 minutes at 30° and deproteinized by boiling. The liquid was passed through cation and anion exchange resins (10 ml. each of Dowex 50 in the H^+ form and Duolite A-2 in the OH^- form). The final volume was 87 ml.

50 ml. were concentrated by evaporation *in vacuo* to 10 ml. and treated with 10 ml. of filtered reagent containing 1 gm. of phenylhydrazine hydrochloride, 1.5 gm. of sodium acetate trihydrate, and a drop of saturated sodium bisulfite. After 30 minutes at room temperature a crystalline precipitate was obtained. This was recrystallized several times from hot 30 per cent ethanol and identified as mannose phenylhydrazone by determining its melting point with the Fisher-Johns apparatus. The phenylhydrazone prepared from the reaction mixture and from pure mannose, as well as a mixture of the two, melted between 199° and 200°.

Additional evidence for the formation of an aldose sugar from fructose was obtained from the reduction of hypoiodite by the incubated mixtures.

The interconversion of fructose and mannose was finally established by incubating enzyme preparations with D-mannose. A rapid appearance of fructose as measured with the Roe reaction was observed. No formation of fructose from glucose could be detected.

With partially purified enzyme, the isomerization of mannose and fructose was studied, starting with either sugar as substrate. Fructose was measured with the Roe reaction. At equilibrium, in 0.1 M Tris-HCl buffer at pH 7.4 and 30°, fructose was found to constitute approximately 71 per cent of the total sugar.

Partial Purification of Enzyme

Partial purification of the isomerase was achieved by ammonium sulfate fractionation of the crude extracts after removal of nucleic acids with protamine. For some experiments, a further purification by adsorption and elution with calcium phosphate gel was used. An arbitrary unit of enzyme activity was established as that amount of enzyme which causes the conversion of 1 μmole of mannose to fructose in 60 minutes at 30° in 0.1 M Tris-HCl buffer at pH 7.4 in the presence of 0.1 M KCl, 0.01 M glutathione, and 0.1 M mannose. Specific activity was expressed as units of enzyme per mg. of protein. A typical procedure of purification is described below.

40 ml. of crude enzyme representing an extract of 8 gm. of cells (wet weight) in 0.1 M Tris-H_2SO_4 buffer, pH 7.4, containing 0.01 M glutathione, were used (total activity, 43,200 units; specific activity, 47.5). 13.3 ml. of SAS were added, and after 10 minutes the precipitate was removed by centrifugation and discarded. 106.7 ml. of SAS were added to the supernatant fluid. After 10 minutes the precipitate was collected and redissolved in 40 ml. of 0.1 M Tris-HCl buffer at pH 7.4 containing 10^{-3} M EDTA. The solution was dialyzed for 60 minutes against demineralized water. 8 ml. of 2 per cent protamine sulfate solution adjusted to pH 5.0 with acetic acid were slowly added with stirring. After 10 minutes, the

precipitate was removed, and the supernatant fluid was dialyzed against 10^{-3} M EDTA for 2 hours. The precipitate was again discarded. To the supernatant solution (62.5 ml.) were added 15.6 ml. of 0.2 M Tris-HCl buffer, pH 7.4, containing 10^{-3} M EDTA and 234 ml. of SAS. After 10 minutes the suspension was centrifuged, and the supernatant solution was discarded. The precipitate was suspended in 10 ml. of 0.2 M Tris-HCl buffer, pH 7.4, containing 10^{-3} M EDTA, and dialyzed for 2 hours against 0.1 M Tris-HCl-EDTA. The precipitate dissolved during dialysis. The solution (30 ml.) was treated with 10 ml. of SAS. After 10 minutes the precipitate was removed by centrifugation and discarded. 20 ml. of SAS were added to the supernatant fluid, and, after 10 minutes, the precipitate was collected, redissolved in 4 ml. of 0.1 M Tris-HCl buffer, pH 7.4, and dialyzed overnight against 10^{-3} M phosphate buffer, pH 6.0, containing 10^{-4} M EDTA. The precipitate was removed, and the solution was diluted with demineralized water to contain approximately 10 mg. of protein per ml. (volume, 8.4 ml.; total activity, 18,500 units; specific activity, 218).

6.3 ml. of aged calcium phosphate gel suspension containing a total of 101.4 mg. of $Ca_3(PO_4)_2$ were added slowly with stirring. After 10 minutes at 0° the gel was removed by centrifugation, and 7 ml. of gel suspension (containing 112.6 mg. of $Ca_3(PO_4)_2$) were added to the supernatant solution. After 10 minutes at 0° the gel was collected by centrifugation and eluted with two successive 8 ml. portions of 0.05 M phosphate buffer, pH 7.4, containing 0.1 M NaCl. After centrifugation, the eluates were pooled (total volume, 16 ml.; total activity, 6,000 units; specific activity, 523).

For most of the experiments to be reported, only ammonium sulfate fractionation was employed. This generally gave a 4- to 6-fold purification and 40 to 70 per cent recovery of the enzyme.

General Properties of Enzyme

No requirement for a heat-stable dissociable cofactor or metal could be demonstrated for the enzymatic activity. Enzyme preparations dialyzed against EDTA or treated with activated charcoal did not show any increased rate of mannose isomerization on the addition of K^+, Na^+, Mg^{++}, Mn^{++}, Ca^{++}, Fe^{++}, Zn^{++}, or Co^{++}, of adenylic acid, adenosine triphosphate, or uridine triphosphate or of boiled crude or partially purified enzyme extracts.

The enzyme was readily inactivated by heavy metals, such as mercury, zinc, or nickel. 50 per cent inhibition of isomerase activity was achieved by incubation for 30 minutes with 7.5×10^{-5} M p-chloromercuribenzoate. The inhibition was completely reversed by the subsequent addition of glutathione. Longer treatment with heavy metals inactivated the isom-

erase irreversibly. Incubation with ferricyanide but not with ferrocyanide also destroyed the activity gradually. 5×10^{-4} M iodoacetate did not inhibit the enzyme.

When fractionation of the isomerase was carried out in Tris-H_2SO_4 buffers in the absence of EDTA or glutathione, it was found that the addition of these compounds or of cysteine had a strong activating effect. This suggested that partial poisoning with heavy metals occurred during the purification.

In early experiments it became apparent that various anions had different effects on the activity of enzyme preparations which were fractionated and dialyzed in the absence of EDTA or glutathione. When enzyme was prepared in dilute Tris-H_2SO_4 buffer, the addition of small amounts of pyrophosphate, chloride, bromide, nitrate, formate, or oxalate had an activating effect which varied in intensity with different batches of enzyme. Phosphate, citrate, and cyanide were generally slightly less effective as activators, while fluoride, thiosulfate, thiocyanate, acetate, lactate, and persulfate either had no effect or were somewhat inhibitory. Enzyme preparations activated by EDTA or glutathione were usually not further activated by chloride. The nature of the cations added together with the anions did not modify the results significantly. No activation was observed with α,α'-dipyridyl. The effect of several selected agents on the rate of mannose isomerization is illustrated in Table I.

When glycylglycine buffers were used, no activation by glutathione, chloride, or EDTA was observed. Glycylglycine is a good chelating agent for heavy metals.

The observations suggested that the enzyme, when prepared without special precautions, may have been partially inactive, owing to poisoning with heavy metals. This view was supported by the observation that the activity of the enzyme in the absence of chloride could be increased by dialysis against EDTA, followed by dialysis against Tris-H_2SO_4 buffer to remove the chelating agent. The activating effect of both chloride and EDTA was greatly diminished by this treatment (see Table II, Enzymes A and B).

Except for the strong activating effect of nitrate ion, which is not generally considered to be a good metal binder, and the lack of activation by dipyridyl, the observations on the effects of anions are compatible with the hypothesis that some sensitive sites of the enzyme are released by the partial combination of the active anions with heavy metals bound to the protein. An attempt to test this hypothesis was made by treating the enzyme with zinc. When a large fraction of the enzyme had been irreversibly inactivated by incubation with the heavy metal and the excess of zinc was removed by dialysis, the activating effect of chloride disappeared, while

TABLE I
Effects of Various Ions on Mannose Isomerase

Experiment No.	Sulfate in Tris-H₂SO₄ buffer, pH 7.4, as μmoles per ml. final reaction mixture	Additions, μmoles per ml. final reaction mixture	Rate, per cent of control without additions
1	2	25 Tris-H₂SO₄*	102
		25 Tris-HCl	140
		25 Tris-HNO₃	144
		25 Tris-formic	137
2	2	10 K₂SO₄	78
		10 KCl	168
		10 KBr	148
		10 KF	106
		10 " + 10 KCl	150
		10 KNO₃	159
		10 K₂HPO₄-KH₂PO₄	132
3	1	1.5 KCl	156
		3.0 "	222
		12 KCl	212
		5 NaCl	212
4	1	12.5 KCl	300
		2.5 glutathione-K	530
		2.5 " + 12.5 KCl	560
		0.5 EDTA	510
		0.5 " + 12.5 KCl	550
5	10	25 NaCl	200
		1 EDTA	222
		1 " + 25 NaCl	222
		5 glutathione-Na	244
		5 " + 25 NaCl	254
6	10	25 NaCl	270
		25 Na₂SO₄	100
		25 NaClO₄	33
		25 " + 25 NaCl	207
		25 KCNS	47
		25 Na₂S₂O₃	84
		25 Na acetate	110
		25 " oxalate	150
		25 " lactate	116
		25 " citrate	186
		1 EDTA	180
		1 dipyridyl	90
		0.1 cysteine	236

Different samples of mannose isomerase prepared in Tris-H₂SO₄ buffer without chelating agents were used in these experiments. The initial rate of fructose production at 30° from 0.1 M mannose was measured in the presence and absence of various added compounds.

* Concentrations expressed as Tris (added as Tris buffers at pH 7.4). Approximately 0.8 equivalent of acid is used for each mole of Tris.

that of EDTA became relatively greater (see Table II, Enzyme C). This may have been due to the complete saturation of certain sensitive sites with the heavy metal, so that they could no longer be released by the partial binding of the metal to chloride. Alternatively, sulfhydryl groups may have become oxidized as a result of the treatment.

Borate buffers decreased the rate of isomerization, probably as a result of the formation of sugar-borate complexes. As will be shown later, the

TABLE II

Rate of Fructose Formation from 0.1 M Mannose

Additions per ml.	μmoles per hr. per ml. original Enzyme A		
	Enzyme A	Enzyme B	Enzyme C
None........................	700	900	70
25 μmoles NaCl................	1250	950	70
1 μmole EDTA.................	1300	1000	260

Enzyme A, a mannose isomerase preparation dialyzed for 26 hours against 0.1 M Tris-H$_2$SO$_4$ buffer, pH 7.4; Enzyme B, the same preparation further dialyzed against 0.1 M Tris-H$_2$SO$_4$ buffer, pH 7.4, containing 1×10^{-3} M EDTA for 15 hours and redialyzed against the same buffer without EDTA for 24 hours; Enzyme C, Enzyme A treated with 1×10^{-4} M ZnCl$_2$ for 30 minutes at room temperature and dialyzed for 8 hours against 0.02 M Tris-H$_2$SO$_4$ buffer, pH 7.4. In all cases the enzyme was diluted with Tris-H$_2$SO$_4$ buffer, pH 7.4, so as to contain 10 μmoles of H$_2$SO$_4$ per ml. in the final reaction mixture. Equal volumes of enzyme and the solutions of various added compounds were incubated for 10 minutes, after which the substrate was added in a volume equal to twice the original volume of enzyme, to give a final concentration of 0.1 M mannose. The preparations were incubated for 30 minutes at 30°. The rates are computed to correspond to the original dialyzed Enzyme A.

equilibrium of the catalyzed reactions is strongly displaced in favor of the ketose sugar when borate is present.

The pH optimum for isomerase activity was found to be approximately 7.5 both in Tris-HCl and phosphate buffers. A pH-activity curve was obtained with solutions containing 0.025 M sodium pyrophosphate, 0.05 M disodium phosphate, and 0.025 M sodium chloride and adjusted to various pH values with phosphoric acid. The maximal rate was at pH 7.5, and the activity decreased to about 75 per cent of the maximum at pH 6.9 and 8.6. At pH 6.0 the activity was about 8 per cent of the maximum.

Glucose had a slight inhibitory action on the reaction. When 0.1 M mannose was used as substrate, a 45 per cent depression of the rate of isomerization was effected by 0.25 M glucose. Mannitol and ribose had practically no inhibitory effect.

Specificity of Mannose Isomerase

It has already been shown that glucose is neither a substrate nor a product of the action of mannose isomerase. A further study with a variety of substrates established that the enzyme is highly specific with regard to the structural configuration of its substrates. The following methods were used to detect transformations of the various aldoses and ketoses tested: (1) the quantitative measurement of the appearance or disappearance of ketohexoses by the method of Roe; (2) chromatography to detect new sugars produced during incubation; and (3) the cysteine-carbazole reaction for keto sugars.

The results are summarized in Table III. The sugars tested as substrates are enumerated in the left-hand column. In those cases in which an isomerization was detected, the catalyzed reaction is shown in the middle column. In the right-hand column are listed those aldose sugars which are apparently not substrates for the enzyme. This list includes the aldoses which were tested with negative results as well as those which could have been products of isomerization of those ketose sugars which were found to be inert experimentally. The sugars which are structurally related by having a common ketose isomer are grouped together by braces.

No isomerization of mannose-6-phosphate was detected with either purified or crude enzyme preparations. Reaction mixtures with this ester did not show the formation of either fructose-6-phosphate or glucose-6-phosphate when tested for the Roe reaction or for the reduction of triphosphopyridine nucleotide with *Zwischenferment*. Phosphoglucose isomerase activity, however, was present in all preparations of mannose isomerase. This could be demonstrated both by the disappearance of fructose-6-phosphate and by the appearance of glucose-6-phosphate when the former compound was used as substrate.

In view of the specificity of the mannose isomerase towards free sugars and its inability to isomerize mannose-6-phosphate, it appeared unlikely that the same enzyme would cause the interconversion of the phosphate esters of glucose and fructose. Further proof of the non-identity of the phosphoglucose isomerase and mannose isomerase was obtained by comparing the rates of the catalyzed reactions in various preparations of the isomerase. An arbitrary unit of phosphoglucose isomerase activity was established as that amount of enzyme catalyzing the disappearance of 1 μmole of fructose-6-phosphate per hour at 30° in 0.1 M Tris-HCl buffer, pH 7.4, containing 10^{-2} M $MgCl_2$. In a crude preparation, the ratio of isomerase to phosphohexoisomerase was found to be 1.7. In five different ammonium sulfate fractions of the same extract the ratio was 2.1, 1.4, 6.5, 10.3, and 9 5, respectively, while for two different phosphate gel eluates the ratios were 3 2 and 1.2. The isomerase preparations of highest specific activity were not

the purest with regard to phosphohexoisomerase. Still further evidence that different enzymes are responsible for the isomerization of fructose and fructose phosphate was the fact that with the latter substrate neither gluta-

TABLE III

Specificity of Mannose Isomerase

Substrates tested	Reaction	Indicated reaction	Aldoses excluded as substrates or products
D-Xylulose	+	D-Lyxose ⇌ D-xylulose	
D-Lyxose	+		
D-Xylose	−		D-Xylose
D-Arabinose	−		D-Arabinose
D-Ribose	−		D-Ribose
L-Xylose	−		L-Xylose
L-Arabinose	−		L-Arabinose
D-Fructose	+	D-Mannose ⇌ D-fructose	
D-Mannose	+		
D-Glucose	−		D-Glucose
D-Tagatose	−		D-Talose
D-Galactose	−		D-Galactose
L-Sorbose	−		L-Idose
			L-Gulose
L-Fructose	−		L-Glucose
			L-Mannose
D-Rhamnose	+	D-Rhamnose ⇌ D-rhamnulose	
L-Rhamnose	−		L-Rhamnose
D-Mannuronic acid	−		D-Mannuronic acid
D-Mannose-6-phosphate	−		D-Mannose-6-phosphate
Sedoheptulose (D-altroheptulose)	+	(?)D-Glycero-D-mannoheptose ⇌ sedoheptulose	
D-Mannoheptulose	−		D-Glycero-D-galaheptose
			D-Glycero-D-taloheptose
L-Glucoheptulose	−		L-Glycero-L-idoheptose
			L-Glycero-L-guloheptose

thione nor EDTA increased the rate of reaction in preparations of mannose isomerase which were activated by these agents.

Reaction with D-Lyxose, D-Rhamnose, and D-Sedoheptulose

The interconversion of lyxose and xylulose was studied, starting with either sugar as substrate. Xylulose was measured with the cysteine-car-

bazole reaction. The initial rate of lyxose disappearance was found to be about one-ninth that of mannose disappearance when these substrates were supplied in a concentration of 0.05 M. At equilibrium the ketose isomer was found to constitute approximately 28.3 per cent of the total pentose.

In agreement with the observations of Cohen (1), it was found that the equilibrium between aldose and ketose is strongly displaced in favor of the latter in the presence of borate. Thus, in 0.025 M pyroborate-HCl buffer at pH 8.0, approximately 48 per cent of a 0.1 M solution of lyxose was converted to xylulose.

The formation of a ketose sugar from D-rhamnose could be demonstrated easily with the cysteine-carbazole reaction. Attempts to separate chromatographically the presumed product of isomerization, 6-deoxy-D-fructose (D-rhamnulose), from D-rhamnose were unsuccessful when either phenol, butanol-acetic acid-water, or butanol-pyridine-water-benzene mixtures were used as solvents. Although some evidence was obtained that the product of isomerization was less susceptible to bromine oxidation than rhamnose, no quantitative assay for it could be based on the reduction of either bromine or hypoiodite. Since no reports on the preparation and properties of D-rhamnulose could be found in the literature, it was necessary to establish that the ketose formed in the reaction was actually 6-deoxy-D-fructose and to obtain a reference standard for the quantitative estimation of the sugar.

The preparation of a syrup containing approximately 55 per cent D-rhamnulose was eventually accomplished, and a crystalline disaccharide derivative of this sugar, D-glucosido-6-deoxy-D-fructoside, was subsequently obtained by the use of sucrose phosphorylase from *P. saccharophila*. The method of preparation and the properties of these new carbohydrates will be described in a separate communication. The structure of the ketose was established by the preparation of the phenylosazone, which was shown to be identical with rhamnosazone. The reducing value of D-rhamnulose for ferricyanide (6) was 102 per cent of an equimolar amount of glucose. The cysteine-carbazole reaction gave a value of 36.2 Klett units per 0.01 μmole of rhamnulose after 2 hours at room temperature, with the No. 54 filter. (0.01 μmole of o-nitrophenylhydrazone of ribulose gave a value of 22.3 under identical conditions.) With a standard available for the cysteine-carbazole reaction, it became possible to evaluate the data obtained in experiments in which rhamnose was used as substrate for the isomerase. It could be calculated that the rate of rhamnulose formation from 0.05 M rhamnose was about one-fourteenth of the rate of fructose formation from mannose under the same conditions. At equilibrium, approximately 37 per cent of the total sugar was present as the ketose. The addition of borate buffer at pH 8.0 was found to displace the equilibrium strongly in favor of the ketose, although the rate of conversion was slightly depressed.

The formation of an aldoheptose from sedoheptulose was demonstrated by the appearance of a new spot on chromatograms of reaction mixtures containing this sugar and the isomerase with butanol-acetic acid-water (4:1:5) as solvent. The new sugar had an R_F value slightly lower than that of sedoheptulose. In addition, a small but significant increase in the hypoiodite reduction was observed after incubation of sedoheptulose with the enzyme. The quantity of aldose formed appeared to be relatively small, and the sugar was not identified. By analogy to the other reactions catalyzed by the isomerase, it seems reasonable to assume that the sugar is D-glycero-D-mannoheptose, rather than the other possible aldose derivative, D-glycero-D-glucoheptose.

<div align="center">DISCUSSION</div>

An analysis of the structure of the various compounds which can or cannot be attacked by the mannose isomerase reveals a rather rigid requirement of the enzyme for the structural configuration of the first 5 carbon atoms of the substrates. A generalized version of the reaction is illustrated.

<div align="center">Aldose   Ketose</div>
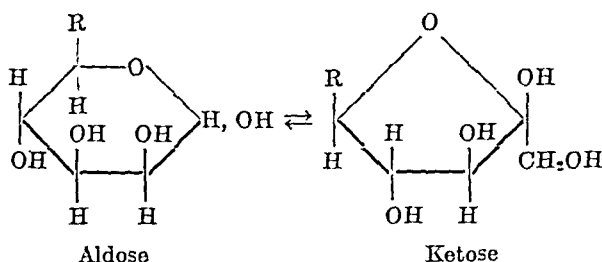
In these formulas, R can be any of the following radicals: —H (in D-lyxose), —CH₃ (in D-rhamnose), —CH₂OH (in D-mannose), —CHOH—CH₂OH (in sedoheptulose). However, apparently it cannot be either —COOH (in mannuronic acid) or a phosphorylated carbinol group, as in mannose-6-phosphate. The enzyme differs from the xylose isomerase, with which it shares a common substrate, xylulose, in the requirement that both the hydroxyl groups of the 2nd and 3rd carbon atoms of the aldose derivative must be above the plane of the ring in the conventional formula. This type of structure in the aldose is also not acceptable for the phosphoglucose isomerases of yeast and of *P. saccharophila* or for the D-arabinose isomerase of *Escherichia coli*. A phosphate group on the terminal carbon atom, which is not tolerated by the mannose isomerase, is required by the mammalian phosphomannose isomerase.

The equilibrium constants found for the isomerization of mannose and lyxose, respectively, are of particular interest. The striking difference between the reactions involving the two structurally similar sugars is that about 71 per cent of mannose is converted to fructose, while only 28 per

cent of the lyxose is isomerized to xylulose. One possible explanation of this discrepancy is that fructose probably exists in solution in both the furanose and pyranose forms, while xyloketose is restricted to the furanose

TABLE IV

Equilibria in Isomerase Reactions

Aldose	Ketose	Possibility of pyranose structure of ketose	Relation of hydroxyl groups on C_2 and C_3 in aldose	K $\dfrac{C \text{ ketose}}{C \text{ aldose}}$	Enzyme*
Pentoses					
D-Arabinose	D-Ribulose	No	Trans	0.18	Arabinose isomerase (1)
D-Xylose	D-Xylulose	"	"	0.19	Xylose isomerase (2)
D-Lyxose	"	"	Cis	0.39	Mannose isomerase
Methylpentoses					
L-Fucose	L-Fuculose	No	Trans	0.14 (pH 8.0)	Arabinose isomerase (1)
D-Rhamnose	D-Rhamnulose	"	Cis	0.58	Mannose isomerase
Hexose phosphates					
D-Glucose-6-phosphate	D-Fructose-6-phosphate	No	Trans	0.47	Phosphoglucose isomerase ((12) p. 304)
D-Mannose-6-phosphate	" "	"	Cis	1.50	Phosphomannose isomerase ((12) p. 299)
Hexoses					
D-Mannose	D-Fructose	Yes	Cis	2.45	Mannose isomerase

* The figures in parentheses refer to the bibliography.

configuration. The aldoses are generally believed to favor strongly the pyranose structure, and it seems probable that it is this form of the aldose which is isomerized. Thus, the two reactions may be written as follows:

(1)   Mannopyranose ⇌ fructofuranose ⇌ fructopyranose

(2)   Xylopyranose ⇌ xyloketofuranose
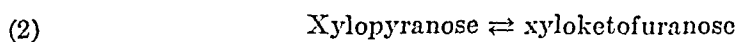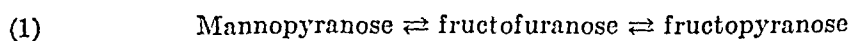
Assuming the aldopyranose-ketofuranose reactions to have the same

equilibrium constants in both cases, one can calculate that approximately 16 per cent of the total fructose is in the furanose form. Gottschalk (11) on the basis of physiological experiments and polarimetric observations has estimated that approximately 20 per cent of dissolved fructose possesses the furanose structure at 20°.

An interesting comparison can be made among several enzymatic reactions involving sugars with regard to the equilibrium between ketose and aldose isomers. In Table IV the available equilibrium constants ($K =$ (C ketose)/(C aldose)) are given, as well as some structural peculiarities of the substrates. It may be noted that in those cases in which the pyranose structure of the ketose is impossible there is a definite correlation between the equilibrium constant and the configuration about the 2nd and 3rd carbon atoms of the aldose isomer. Those aldoses which have the hydroxyl groups in the trans position are favored over those having the cis configuration. Thus, less lyxose than xylose is produced from xylulose, and less mannose-6-phosphate than glucose-6-phosphate is formed from fructose-6-phosphate. The high value of the equilibrium constant for the isomerization of mannose-6-phosphate cannot be ascribed to the furanose-pyranose isomerization of the ketoses. It must be borne in mind, however, that very little information is available on the occurrence of hydrates and the various ring forms of the aldose sugars or their phosphate esters. Evidence for the occurrence of both L-arabopyranose and L-arabofuranose may be adduced from the observation that in aqueous solution arabinose can be oxidized to δ-arabonolactone with bromine (13) or to γ-arabonolactone with an arabinose dehydrogenase from *P. saccharophila* (14).

SUMMARY

1. A new enzyme, mannose isomerase, has been found in a fructose-utilizing mutant strain of *Pseudomonas saccharophila* and has been partially purified.

2. The enzyme catalyzes the interconversion of D-mannose and D-fructose, D-lyxose and D-xylulose, and D-rhamnose and D-rhamnulose (6-deoxy-D-fructose). An aldose, presumably D-glycero-D-mannoheptose, is produced from sedoheptulose.

3. The isomerase could not be shown to require any organic cofactors or metallic ions tested or present in crude preparations. It was very sensitive to poisoning with heavy metals and slowly inactivated with ferricyanide.

An activating effect of sulfhydryl compounds, chelating agents, and certain anions was observed. The pH optimum was 7.5.

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BIOSYNTHESIS OF ARGININE FROM CANAVANINE AND ORNITHINE IN KIDNEY

By JAMES B. WALKER*

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Borsook and Dubnoff (1, 2) and Bloch and Schoenheimer (3) have demonstrated that the amidine moiety of arginine is the precursor of the corresponding group of glycocyamine; in mammals this reaction is carried out primarily in the kidney. Fuld (4) has recently observed that the arginine-glycine transamidination reaction is reversible. By means of group transfer the high chemical potential of the amidine moiety is conserved. Since other metabolites containing the guanidino group occur in nature, it is of interest to determine whether they also participate in transamidination reactions. One such compound that might be investigated is canavanine, a naturally occurring amino acid which possesses a terminal guanidinoxyl group.

In this paper data will be presented which show that in the presence of an enzyme from hog kidney canavanine donates an amidine group to ornithine, with the formation of arginine and canaline.

Materials—L-Canavanine sulfate, L-ornithine dihydrochloride, L-arginine monohydrochloride, and beef liver arginase were purchased from the Nutritional Biochemicals Corporation. Preliminary experiments were carried out with canavanine isolated from jack bean meal as the thrice recrystallized flavianate; canavanine was regenerated from the flavianate with barium hydroxide.

Analytical Methods—The ascending paper chromatographic procedure of Williams and Kirby was employed (5, 6). Paper chromatograms were sprayed with ninhydrin to detect primary amino groups or alkaline ferricyanide-nitroprusside to detect guanidino groups (6). Since canavanine does not react significantly with the Sakaguchi reagent, arginine could be determined colorimetrically in the reaction mixture with this reagent. The modified procedure of Albanese and Frankston (7) was employed. The components were mixed at 5°, and the optical densities at 540 mμ were read within 10 minutes with an Evelyn colorimeter. The stipulated hypochlorite concentration was met by using commercial Clorox, diluted 1:1.

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Results

Since mammalian kidney was already known to contain an enzyme which catalyzes a reversible arginine-glycine transamidination reaction, it was logical that hog kidney should be the tissue first examined for activity in a canavanine-ornithine transamidination. The low arginase activity of mammalian kidney was considered to be another favorable characteristic of this tissue, as far as interpretation of results was concerned. Although it was probable that such a transamidination, if it occurred, would be reversible, analytical considerations indicated that the reaction might be most conveniently studied in the following direction:

$$
\underset{\substack{|\\ \text{NH}_2}}{\text{HOOC—CH}}\text{—CH}_2\text{—CH}_2\text{—O—NH—}\overset{\substack{\text{NH}\\ \|}}{\text{C}}\text{—NH}_2 + \tag{1}
$$

Canavanine

$$
\text{H}_2\text{N—CH}_2\text{—CH}_2\text{—CH}_2\text{—}\underset{\substack{|\\ \text{NH}_2}}{\text{CH}}\text{—COOH} \rightarrow
$$

Ornithine

$$
\underset{\substack{|\\ \text{NH}_2}}{\text{HOOC—CH}}\text{—CH}_2\text{—CH}_2\text{—O—NH}_2 +
$$

Canaline

$$
\text{H}_2\text{N—}\overset{\substack{\text{NH}\\ \|}}{\text{C}}\text{—NH—CH}_2\text{—CH}_2\text{—CH}_2\text{—}\underset{\substack{|\\ \text{NH}_2}}{\text{CH}}\text{—COOH}
$$
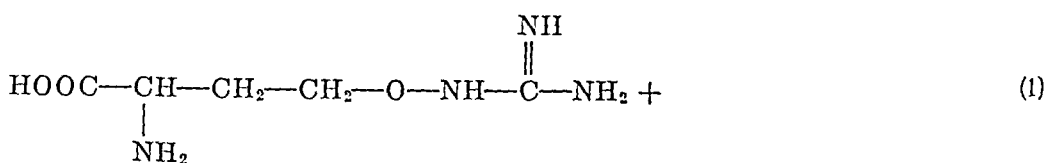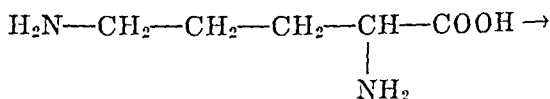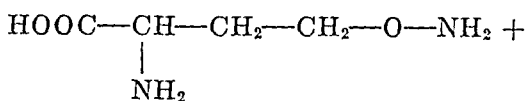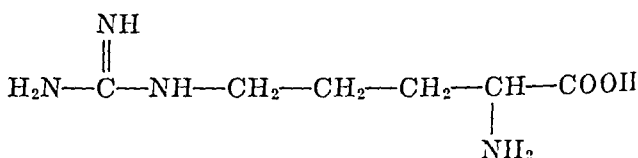
Arginine

With this postulated reaction under consideration, the search for an active enzyme preparation was started.

Enzyme Preparation—Acetone-dried hog kidney (8) was extracted for 2 hours with 0.05 M potassium phosphate buffer, pH 7.4, at 5°. Protein was precipitated by the dropwise addition of a saturated solution of ammonium sulfate to 60 per cent saturation. The precipitate was redissolved in 0.02 M buffer, and the solution was added dropwise with stirring to 50 volumes of cold acetone. The precipitate was collected by suction filtration, washed with acetone, and the acetone removed *in vacuo*. It was found by paper chromatographic analysis, and confirmed by the Sakaguchi test for arginine, that this preparation catalyzed the reaction

as postulated above. In an attempt to concentrate the activity, the acetone powder was dissolved in 0.05 M phosphate buffer, pH 7.0, and refractionated with ammonium sulfate. A clean fractionation of the enzymatic activity was not achieved in this manner, but the fraction precipitating between 30 and 40 per cent saturation had the highest specific activity. Dialysis of this fraction for 24 hours at 3–5° against three changes of 400 volumes of 0.01 M phosphate buffer, pH 7.0, resulted in a gain, rather than a loss, in activity. Similarly, the addition of the chelating agent ethylenediaminetetraacetate increased the activity slightly. Consequently subsequent dialyses were carried out against buffer plus ethylenediaminetetraacetate. After dialysis, an acetone powder of the 30 to 40 per cent fraction was prepared and stored in the cold.

Reaction Characteristics—Activity of the dialyzed preparations was not enhanced by the addition of cysteine, magnesium, manganese, or concentrates of liver or yeast coenzymes (Sigma). The reaction proceeds at a significant rate between pH 6.2 and 8.2, with an optimum at pH 7.2 under the conditions employed.

The effect on the reaction of omitting one of the components is shown in Table I. It can be seen that the reaction does not proceed unless all components are present. If the enzyme is inactivated by heating the reaction mixture for 5 minutes at 100°, no arginine is formed upon subsequent incubation. Lysine will not substitute for ornithine in this reaction; the results obtained with glycine will be discussed later. Although the reaction products could be detected after 2 hours incubation at room temperature, in this experiment the reaction mixtures were incubated for 43 hours, 11 hours after the arginine concentration had reached its maximal value in Reaction Mixture 1. This value represents a 42 per cent conversion of ornithine into arginine. In Reaction Mixture 5, in which the canavanine concentration was doubled, the conversion was increased to 67 per cent. Whether the arginine concentration obtained in Reaction Mixture 1 represents the equilibrium value under these conditions will not be known until a more purified enzyme preparation is available.

Identification of Reaction Products—The fact that the reaction product which gives a red color with the Sakaguchi reagent is indeed arginine was confirmed by paper chromatography, in which either a ninhydrin spray was employed for the primary amino group or an alkaline ferricyanide-nitroprusside spray to detect the guanidino moiety (6). For this purpose the most satisfactory solvent system was water-saturated phenol, with an ammonia atmosphere. Arginine spots were obtained only from those reaction mixtures in which all components were present. Additional confirmation was furnished by microbiological assays for arginine with *L. mesenteroides* P-60 (Table I). This organism was chosen because it

responds to arginine and not to ornithine or citrulline (9), since it lacks the enzyme argininosuccinase (10). Of particular importance for the purpose of this assay is the fact that the growth of this organism is not affected by canavanine (9). Consequently in a reaction mixture which contains canavanine, ornithine, canaline, and arginine, *L. mesenteroides*

TABLE I

Results of Colorimetric and Microbiological Assays for Arginine Formation

Each tube contained 15 mg. of an acetone powder of a dialyzed, 30 to 40 per cent ammonium sulfate fraction of kidney extract as the enzyme source, plus 1.6 ml. of 0.125 M potassium phosphate buffer, pH 7.0, and other components as shown below. Incubation at room temperature, under toluene, for 43 hours, 11 hours after maximal arginine production was obtained. The reactions were stopped by heating at 100° for 3 minutes and the mixtures centrifuged. Analyses of supernatant solutions for arginine were carried out both colorimetrically with the Sakaguchi reagent against an arginine standard and microbiologically with *Leuconostoc mesenteroides* P-60.

Reaction mixture No.	Substrates	Arginine produced	
		Colorimetric assay	Microbiological assay
		$\mu moles$	$\mu moles$
1	Canavanine, 76 μmoles; ornithine, 78 μmoles	33	32
2	" 76 "	4	2
3	Ornithine, 78 μmoles	0	1
4	Canavanine, 76 μmoles; lysine, 78 μmoles	3	3
5	" 153 μmoles; ornithine, 78 μmoles	52	53
6	" 76 μmoles; ornithine, 78 μmoles; boiled 5 min. at 0 time	3	2
7	Canavanine, 76 μmoles; glycine, 80 μmoles	16*	3
8	Glycine, 80 μmoles	1	1

* The Sakaguchi color is reported in arginine equivalents, but, as the microbiological assay and paper chromatography indicate, the color is due not to arginine, but to glycocyamine.

responds only to arginine. It can be seen from Table I that the colorimetric and microbiological assays for arginine are in close agreement.

The other reaction product, canaline, was identified by a specific and sensitive paper chromatographic method developed in this laboratory. In this method advantage was taken of the fact that canaline is an *O*-substituted hydroxylamine capable of reacting with carbonyl groups (11), with an α-amino group which can be detected with ninhydrin. Consequently the R_F values of the oxime type complexes of canaline with various carbonyl compounds should serve to identify canaline in complex reaction mixtures. Preliminary experiments were performed to establish

the corresponding R_F values. Beef liver arginase was incubated with canavanine to form canaline. After the reaction was complete, various carbonyl compounds were added to aliquots of the deproteinized solution. The solutions were chromatographed on paper with a solvent of water-saturated phenol, ammonia atmosphere, and sprayed with ninhydrin. The R_F values of the complexes of canaline with several carbonyl compounds are listed in Table II. In an adjacent column are given the R_F values of the ninhydrin spots obtained when the same carbonyl compounds were added to aliquots of the deproteinized canavanine-ornithine reaction mixture. These latter spots were not observed when either canavanine,

TABLE II

Canaline Complexes with Various Carbonyl Compounds

The solvent system is water-saturated phenol, ammonia atmosphere, and the color reagent is ninhydrin, which reacts with the α-amino group of the canaline moiety. The middle column gives the results obtained by adding the carbonyl compound to a solution of authentic canaline; the last column, the corresponding R_F values for the new ninhydrin-reactive compound obtained with the complete reaction mixture after incubation. These results show that canaline is a product of the canavanine-ornithine transamidination reaction.

Carbonyl compound added		R_F value of oxime	
		Canaline	Reaction mixture
Ribose-5-phosphate	.....	0.05	0.04
Ribose	...	0.43	0.43
Pyruvate	.. ..	0.40	0.38
Oxalacetate	.	0.11	0.10
α-Ketoglutarate	...	0.19	0.20

ornithine, or the enzyme was omitted during incubation. From these data it is apparent that canaline is a product of the transamidination reaction. Further confirmation was obtained by isolating the complexes obtained with pyruvate and α-ketoglutarate by means of paper chromatography. The respective paper strip eluates were subjected to treatment with palladium black and hydrogen gas at room temperature and atmospheric pressure for 3 hours and were rechromatographed on paper. It was found that the pyruvate complex yielded homoserine and alanine, while the α-ketoglutarate complex yielded homoserine and glutamic acid, as determined by their R_F values in solvents of water-saturated phenol, ammonia atmosphere, and butanol-acetic acid-water (4:1:1). These are the products which would be expected from a reduction of an oxime linkage between canaline and the respective keto acid.

Reversibility of Reaction—That the canavanine-ornithine transamidina-

tion reaction is reversible was indicated by the results obtained in the following experiment. A mixture of 25 mg. of canavanine sulfate and 3 ml. of 0.1 M phosphate buffer was adjusted to pH 7.5, and 15 mg. of arginase were added. The mixture was incubated in the presence of toluene for 23 hours at room temperature to assure the complete hydrolysis of canavanine to canaline, and then heated for 5 minutes at 100°. The mixture was centrifuged to remove coagulated protein. To 1.2 ml. of the canaline-containing supernatant solution were added 20 mg. of L-arginine monohydrochloride and 10 mg. of the dialyzed transamidinase preparation. After incubation for 20 hours at room temperature in the presence of toluene, the reaction mixture was deproteinized by heating at 100° for 3 minutes. Paper chromatograms of the supernatant solution, with water-saturated phenol, ammonia atmosphere, as the solvent, were sprayed with alkaline ferricyanide-nitroprusside reagent. Below the red arginine spot, at the predicted R_F value, could be distinguished the violet spot characteristic of canavanine. This spot was not present in the canavanine hydrolysate before arginine and enzyme were added. No canavanine was formed when either arginine or transamidinase was omitted from the reaction mixture.

Evidence for Canavanine-Glycine Transamidination—Preliminary experiments indicate that glycine also can act as an amidine acceptor when canavanine is the donor. In the experiment described in Table I, when glycine was substituted for ornithine, it was observed that a compound was produced which reacted in the Sakaguchi test, but did not support growth of *L. mesenteroides* in a medium lacking only arginine. Paper chromatographic analysis, with butanol-acetic acid-water (4:1:1) as solvent and an alkaline ferricyanide-nitroprusside spray, provided strong evidence that the compound produced was glycocyamine. This compound was not produced in the absence of canavanine. When the compound was eluted and hydrolyzed with alkali, a ninhydrin-reactive compound was formed which corresponds with glycine on paper chromatograms.

DISCUSSION

It is not known at present whether canavanine and canaline are normal metabolites of higher animals. However, it is known that plants containing canavanine are ingested by higher animals. Consequently the metabolic pathways in which canavanine can participate are of interest. In mammals, canavanine may combine with fumarate to form canavaninosuccinic acid (12), be hydrolyzed to canaline and urea (13), or undergo transamidination, with the formation of canaline. It is interesting to speculate about the rôle of this transamidination reaction in metabolism. Ap-

parently *L. mesenteroides* lacks such an enzyme, since its arginine requirement cannot be met by a combination of canavanine and ornithine. If avian kidney can perform this transamidination, then in those birds which cannot synthesize citrulline from ornithine it is possible that canavanine might replace arginine in the diet, provided that sufficient endogenous or exogenous ornithine is present.

The mechanism of the canavanine-ornithine transamidination reaction reported here is not known. A cofactor requirement was not shown by these experiments. Since arginine-handling enzymes of many organisms apparently cannot distinguish completely between arginine and canavanine, it is tempting to speculate that reactions similar to the following may occur.

$$\text{Canavanine} + \text{enzyme} \rightleftharpoons \text{enzyme} \sim \text{urea} + \text{canaline} \qquad (2)$$

$$\text{Enzyme} \sim \text{urea} + \text{ornithine} \rightleftharpoons \text{arginine} + \text{enzyme} \qquad (3)$$

$$\text{Sum.} \quad \text{Canavanine} + \text{ornithine} \rightleftharpoons \text{arginine} + \text{canaline} \qquad (4)$$
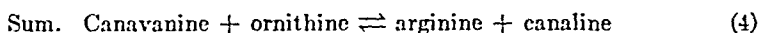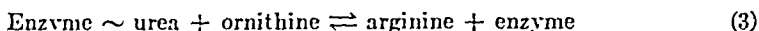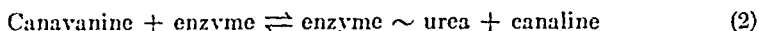
In this interpretation of the results reported in this paper, a single enzyme would be involved in the activation of both arginine and canavanine, and the same enzyme-amidine complex would be formed from either substrate. If this scheme is correct, the amidine group of arginine may be considered to be in a state of dynamic metabolic equilibrium in mammalian kidney, as well as in other biological systems which contain this enzyme (Reaction 3).

The postulated enzyme-amidine complex may be considered to be a form of "active urea." If such a complex is formed, it is possible that in some organisms a mechanism might exist for the synthesis of that complex from urea plus an energy source. A similar mechanism was earlier postulated to account for the utilization of urea as the sole nitrogen source for growth of organisms which apparently lack urease (14). Because of the wide-spread use of urea in foliar sprays and as a nitrogen source for ruminants, such a proposal warrants further study.

As an interesting by-product of this investigation, it might be noted that the reaction of canaline with various carbonyl compounds of biochemical interest could serve as a useful means of identification of those carbonyl compounds in complex biological mixtures. Canaline can be readily formed from commercially available canavanine and arginase. The procedure would involve simply adding the canavanine hydrolysate, containing canaline, to the unknown mixture and chromatographing the solution on paper. Use of the the sensitive ninhydrin reagent would permit the identification of small quantities of such compounds as pyruvate, α-ketoglutarate, oxalacetate, and ribose (Table II).

SUMMARY

An enzyme preparation from hog kidney was found to catalyze a transfer of the amidine moiety of canavanine to ornithine, with the formation of arginine and canaline. No cofactor requirement was observed; the optimal pH is 7.2. Preliminary experiments indicated that the reaction is reversible. The same crude enzyme preparation apparently catalyzes a canavanine-glycine transamidination, glycocyamine being tentatively identified as a reaction product. It is suggested that the canavanine-ornithine transamidination reaction involves the reversible formation of the same enzyme-amidine ("active urea") complex from both canavanine and arginine. If this concept is correct, the amidine group of arginine may be considered to be in a state of dynamic equilibrium in mammalian kidney.

As a by-product of this investigation, a previously unreported method for identifying carbonyl compounds of biological interest was described. An arginase hydrolysate of canavanine, containing canaline, a ninhydrin-reactive O-substituted hydroxylamine, is added to the unknown to be identified, and the resulting solution is chromatographed on paper. The oxime type complexes formed between canaline and such compounds as pyruvate, α-ketoglutarate, and oxalacetate can be readily identified by their characteristic R_F values, as detected with ninhydrin.

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THE BIOCHEMISTRY OF HYDROGENOMONAS

IV. THE INHIBITION OF HYDROGENASE BY OXYGEN*

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Hoberman and Rittenberg (1) found that the hydrogenase activity of intact *Proteus vulgaris* cells (assayed by deuterium exchange) was not affected by cyanide added anaerobically, but was sensitive to cyanide added in the presence of oxygen. It was suggested that a prosthetic group of hydrogenase is reversibly oxidized by molecular oxygen to an inactive form which reacts irreversibly with cyanide. This cyanide effect constitutes the main evidence for the view that the prosthetic group is an iron porphyrin compound. In addition, carbon monoxide inhibition is partially reversed by light in *Proteus* (1), although not in *Escherichia coli* (2, 3) or *Azotobacter vinelandii* (4). Nitric oxide inhibition (5, 6) has been considered further evidence for iron porphyrin participation (5). Inhibition of hydrogenase by reversible oxygenation has also been proposed (7).

The type of interaction between cyanide and oxygen reported by Hoberman and Rittenberg has been observed in measurements of hydrogen uptake in the presence of an electron acceptor by intact cells of *E. coli* (2) and *A. vinelandii* (8) and in intact cells (9, 10) and cell-free preparations (10) of *Hydrogenomonas facilis*. Participation of an iron porphyrin prosthetic group has, however, been questioned on various grounds (3, 9–11), among them the high levels of cyanide usually required for inhibition. It has also been suggested that the hydrogenase of *Clostridium pasteurianum* is a molybdenum flavoprotein (12).

This paper reports a reinvestigation of the effects of oxygen and cyanide on hydrogenase activity. Intact cells of *H. facilis* were used. The results suggest that oxygen is the inactivating agent, and that cyanide acts indirectly by inhibiting an enzyme which decomposes the inactive hydrogenase-oxygen compound or complex.

Methods

Suspensions of autotrophically grown *H. facilis* cells were used. The cells were harvested after 40 to 60 hours growth on inorganic agar plates under hydrogen, oxygen, and carbon dioxide (10). Cell weights were esti-

mated turbidimetrically. Hydrogenase activity was assayed manometrically with MB[1] as acceptor. Gas mixtures were made up volumetrically in plastic bags and introduced into individual Warburg flasks by evacuating them three times to 0.1 atmosphere and filling. When all of the flasks in an experiment required the same gas mixture, it was made up manometrically directly in the flasks by connection to a manifold. Atmospheres of nominally pure hydrogen were obtained by evacuating the flasks 5 times to 0.1 atmosphere and filling them from a commercial tank. Solutions of potassium cyanide were unbuffered to minimize loss of HCN by volatilization; hence some evolution of HCN occurred on addition of these solutions to the buffered (pH 7) cell suspensions. The change in pH following addition of cyanide was less than 0.2 unit, and its effect on hydrogenase activity was negligible.

Results

At a constant partial pressure of hydrogen and constant levels of cyanide and MB, gas uptake by intact cells of *H. facilis* was progressively inhibited as the partial pressure of oxygen was increased (Fig. 1). Results of a preliminary experiment in which the gas phases were made up by mixing hydrogen and air (so that the hydrogen content decreased as the oxygen increased) are also plotted in Fig. 1. The close agreement indicates that under the conditions of these experiments oxygen concentration is the principal factor determining hydrogenase activity and that variation in hydrogen concentration within the range tested has little effect. In these experiments the cells were incubated with cyanide under the experimental atmosphere for 30 minutes before addition of MB.

Despite the simple curve of Fig. 1, the situation was rather complex, since oxygen affected the system in other ways than by inactivating hydrogenase (Fig. 2). The positive slope of the control curve in the region of low oxygen concentration was due primarily to reaction of oxygen with MBH$_2$. The approximately level corresponding portion of the curve for the cyanide series presumably resulted from a similar increase in oxygen uptake which masked the decrease in hydrogen utilization. In addition, the natural mechanisms transferring electrons to oxygen, which are inhibited by cyanide, may have participated significantly in the control flasks.

In the experiments just described, both the concentration of cyanide and the time of preincubation with cyanide before addition of MB were held constant. For study of these variables, a constant oxygen partial pressure of approximately 0.1 atmosphere was used. Under this atmosphere, varying amounts of potassium cyanide were added and the suspensions in-

---

[1] MB and MBH$_2$ denote the oxidized and reduced forms, respectively, of methylene blue.

cubated for 30 minutes before addition of MB. Some loss of HCN by volatilization inevitably occurred, but was minimized by the fact that the potassium cyanide solution in the side arm was unbuffered and the flasks were evacuated only once to 0.5 atmosphere and immediately filled with hydro-
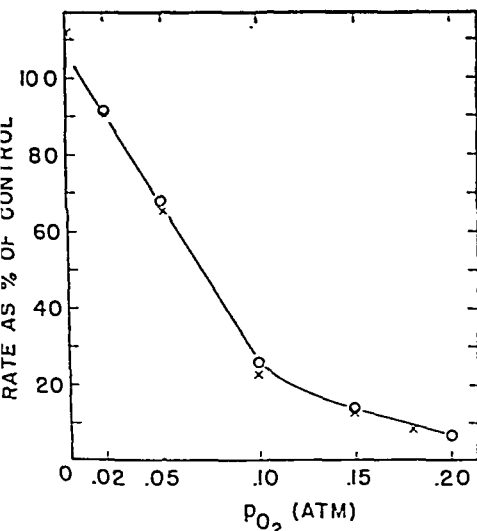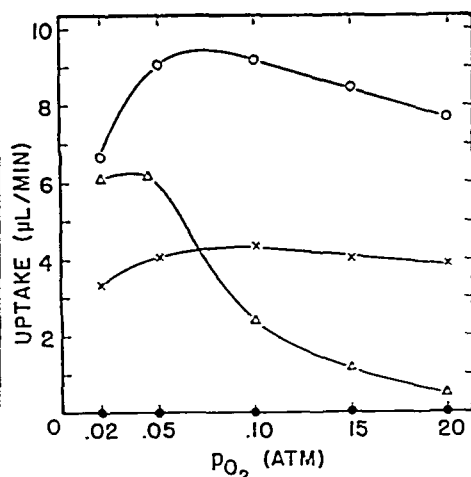
FIG. 1   FIG. 2

FIG. 1. Effect of oxygen partial pressure on rate of gas uptake by autotrophically grown *H. facilis* cells with MB as electron acceptor. Each flask contained 0.3 mg. (dry weight) of cells in 2 ml. of 0.07 M phosphate, pH 7.0. MB and KCN, 25 μmoles (1.25×10^{-2} M) each. O, gas phase 0.4 atmosphere of hydrogen plus the indicated partial pressure of oxygen, made up to 1 atmosphere with helium; ×, gas phase air at 5 times the indicated pressure of oxygen, made up to 1 atmosphere with hydrogen. KCN was added after the flasks were filled with the experimental atmosphere, and MB was added 30 minutes later.

FIG. 2. Interaction between oxygen and cyanide in inhibiting hydrogenase of *H. facilis*. Conditions as described for curve (O) of Fig. 1. O, control; MB added after 30 minutes incubation under the indicated atmosphere; △, MB added after 30 minutes incubation with KCN under the indicated atmosphere; ×, hydrogen-oxygen reaction in absence of MB and KCN; ●, hydrogen-oxygen reaction in presence of KCN.

gen. The results (Fig. 3) show that cyanide concentration may be varied over a considerable range with little effect on the degree of inhibition. At all levels tested (from 8×10^{-5} M to 2×10^{-2} M) cyanide completely prevented the hydrogen-oxygen reaction during the 30 minutes before addition of MB. In parallel experiments, cyanide at none of these levels significantly affected the rate of MB reduction under pure hydrogen.

Changes in the length of the interval during which the cells were incubated with cyanide before addition of MB also failed to affect the degree of

inhibition (Fig. 4). When MB was added 10, 20, or 40 minutes after addition of cyanide, initial gas uptake amounted to 35, 33, and 35 per cent, respectively, of the rate of the corresponding control suspension lacking cyanide. Furthermore, the actual rates as well as the ratio of inhibited to control rates were unchanged. While the initial rate in the flask receiving
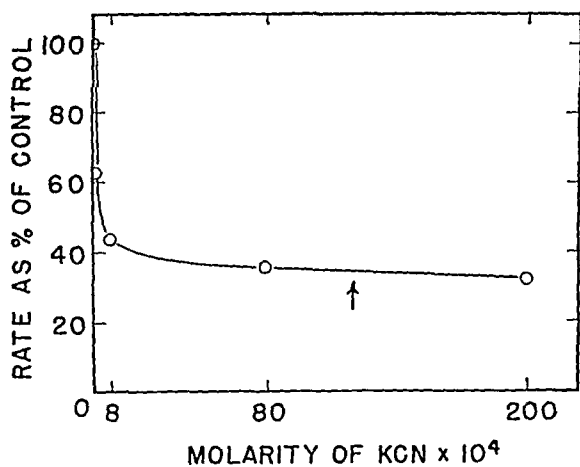
FIG. 3. Effect of cyanide concentration on gas uptake from an atmosphere of 50 per cent hydrogen and 50 per cent air by autotrophically grown *H. facilis* cells with 1.25×10^{-2} M MB as electron acceptor. 0.2 mg. (dry weight) of cells in 2 ml. of 0.07 M phosphate, pH 7.0. The control rate was 5.4 μl. per minute. The arrow indicates the cyanide level used in other experiments reported in this paper.
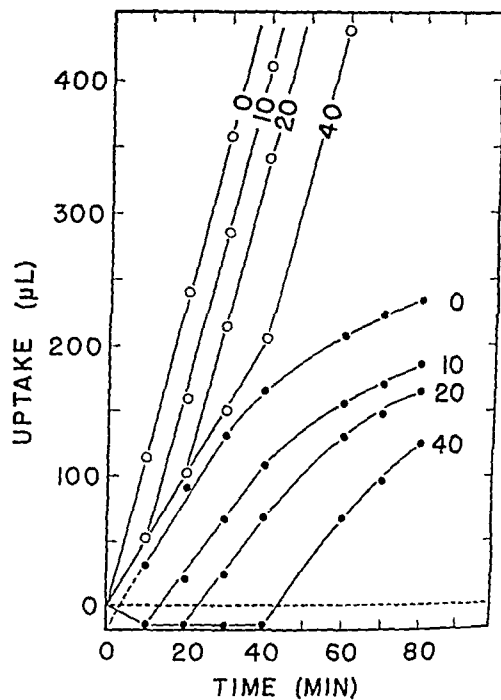
FIG. 4. Effect of time of incubation with KCN and oxygen on the rate of gas uptake from an atmosphere of 50 per cent hydrogen and 50 per cent air by autotrophically grown *H. facilis* cells with MB as electron acceptor. Each flask contained 0.3 mg. (dry weight) of cells in 2 ml. of 0.07 M phosphate, pH 7.0. 1.25×10^{-2} M MB added at the times indicated by the figures on the curves. O, control; ●, 1.25×10^{-2} M KCN added at 0 time.

cyanide and MB simultaneously cannot be determined precisely because of interference by equilibration of HCN partial pressure with the buffered suspension, the rate was slightly higher than in the other flasks. Evidently oxygen inactivation of hydrogen uptake in the presence of cyanide and in the absence of electron acceptor reaches a steady state within 10 minutes and remains constant for at least an additional 30 minutes. The 0 time curves of Fig. 4 resemble closely the results reported by Schlegel (9) for a similar experiment in which all mixing was at 0 time.

Since the degree of inhibition is relatively independent of cyanide and hydrogen concentrations, it appeared that the steady state might be definable in terms of oxygen concentration, with cyanide being necessary for manifestation of the inhibition but not directly involved. Fig. 1 supplies the data for such a treatment. If total hydrogenase is designated by (E_T) and active hydrogenase by (E), the ratio $(E)/(E_T)$ may be approximated by the ratio of reaction rates in the presence and in the absence of cyanide. If, then, the assumption is made that oxygen reacts with the enzyme according to the reaction

$$E + n\ O_2 \rightleftharpoons E(O_2)_n \tag{1}$$

where $E(O_2)_n$ represents an inactive compound or complex that accumulates only in the presence of cyanide, the equilibrium constant will be given by

$$K = \frac{[E(O_2)^n]}{(E)(O_2)^n} \tag{2}$$

By substituting $[E(O_2)_n] = (E_T) - (E)$, Equation 2 becomes

$$K = \left[\frac{(E_T)}{(E)} - 1 \right] \frac{1}{(O_2)^n} \tag{3}$$

When the values of (O_2) and $(E)/(E_T)$ from the experiments summarized in Fig. 1 are substituted in Equation 3, the value of K is essentially constant when $n = 2$ (K = about 280). This result indicates an over-all second order dependence of steady state inactivation on oxygen concentration. While the simplest explanation is that 2 molecules of oxygen per active site on the hydrogenase molecule are required for inactivation, other mechanisms are possible in view of the multiple rôle of oxygen in this system (Fig. 2).

It will be noted (Fig. 4) that, besides the steady state inactivation of hydrogenase, there is a progressive inhibition in the cyanide series which first becomes evident 20 to 40 minutes after addition of MB. Gas uptake in control flasks was linear as far as measured (to about 700 μl.). This effect suggests inhibition by a product of the reaction in the presence of cyanide. Since MBH_2 does not inhibit under hydrogen, it seems possible that hydrogen peroxide formed by reaction of MBH_2 with oxygen may be the inhibiting compound. Preliminary experiments indicate that added peroxide inhibits hydrogenase when catalase is inactivated by cyanide. The alternative explanation, that cyanide inhibits hydrogenase directly but only during enzyme function, is unlikely in view of the absence of a comparable progressive inhibition when oxygen is not present.

DISCUSSION

The evidence presented seems incompatible with a reversible oxidation of hydrogenase, followed by irreversible reaction of the oxidized form with cyanide (1). In such a case the inhibition should be nearly complete when both oxygen and cyanide are present at relatively high levels, since reaction with cyanide would remove the oxidized enzyme and force the oxidation to completion. However, with oxygen at 0.1 atmosphere and cyanide at 0.02 M, the rate of gas uptake was still over 30 per cent of the control rate (Fig. 3).

The shape of the curve for inactivation as a function of cyanide concentration (Fig. 3) would also appear to rule out participation of an iron porphyrin compound directly as a prosthetic group of hydrogenase. On the other hand, the results are consistent with the view that an iron porphyrin enzyme is involved in the protection of hydrogenase by decomposition of an inactive hydrogenase-oxygen compound.

The hypothesis suggested by the results presented here may be summarized as follows: Hydrogenase reacts with or adsorbs oxygen to give an inactive form of the enzyme. Normally, and especially in the presence of hydrogen, the inactivated form does not accumulate to a measurable degree because of rapid enzymic decomposition. In the presence of cyanide, this protective mechanism is inhibited and the inactive form of hydrogenase accumulates to a level determined by the concentration of oxygen and presumably of cyanide-insensitive systems catalyzing reactivation. Because the inactivated form is decomposed at a finite rate even in the presence of cyanide, removal of oxygen will lead to reactivation of the enzyme. It does not seem likely that reactivation occurs to any considerable degree by uncatalyzed dissociation of the enzyme-oxygen compound, in view of the relatively slow rate of reactivation of cell-free preparations. Either the dilution involved in preparation of the extract or loss of spatial orientation between hydrogenase and the protecting enzyme might explain this effect.

The inactive compound might be an enzyme peroxide, the protecting enzyme be capable of decomposing the peroxide, and the non-enzymic catalysts such ions as ferrous. If an electron carrier is involved in MB reduction, it may be the site of oxygen inhibition. However, the similarity between the cyanide effect reported here and that observed with the exchange reaction (1) suggests that the same mechanism is involved in both cases, and it seems likely that the site of oxygen inactivation is hydrogenase itself.

The hypothesis presented appears to be compatible not only with the results reported here, but also with earlier reports of cyanide and oxygen effects on hydrogenase activity in various species (2, 3, 8, 9). Of these, only one requires special comment. Joklik (3) found that on a percentage

basis the inhibition of cell-free *E. coli* hydrogenase by aerobically added cyanide was intensified by incubation under hydrogen. This finding was considered to demonstrate stronger cyanide inhibition under hydrogen than under air, in contrast to the results obtained in all other cases. The preparation used by Joklik was largely inactive before addition of cyanide, however, and both the cyanide-containing and the control preparations were reactivated by incubation under hydrogen. The increase in per cent inhibition reflected in reality a slower reactivation of the cyanide-containing preparation, a result predicted by the hypothesis outlined here.

In the presence of hydrogen and the absence of cyanide the hydrogenase activity of intact *H. facilis* cells is not reduced by oxygen at any level tested (up to 20 per cent). Fisher, Krasna, and Rittenberg (7) reported that the rate of the deuterium exchange reaction carried out by intact cells or cell-free preparations of *Proteus* is decreased by oxygen. In the case of cell-free extracts, oxygen concentrations well below 1 per cent were markedly effective, even in the presence of 95 per cent hydrogen. It is not clear whether this difference in the level of oxygen required for inhibition reflects a species difference or results from the comparison of intact cells with cell-free preparations.

SUMMARY

Cyanide at 2×10^{-2} M fails to inhibit hydrogenase activity of intact cells of *Hydrogenomonas facilis* (estimated by methylene blue reduction under a hydrogen atmosphere), but the hydrogen-oxygen reaction is abolished by the same inhibitor at 8×10^{-5} M. Under a hydrogen-oxygen atmosphere, the rate of hydrogen uptake in the presence of methylene blue is nearly independent of cyanide concentration above 8×10^{-4} M, and within rather wide limits is independent of hydrogen concentration and of time of preincubation with cyanide. Under these conditions, the degree of inhibition depends almost exclusively on the oxygen concentration. Oxygen at the levels tested does not inhibit in the absence of cyanide.

These results suggest that hydrogenase forms an inactive compound or complex with oxygen which is decomposed by some cyanide-sensitive enzyme, and which accumulates to detectable levels only in the presence of cyanide. According to this view, there is no direct effect of cyanide on hydrogenase. Effects of cyanide and oxygen on hydrogenase previously reported are discussed in terms of this hypothesis.

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16-SUBSTITUTED STEROIDS

XVI. 5-ANDROSTEN-3β-OL-16-ONE

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Dehydroepiandrosterone (5-androsten-3β-ol-17-one) is a normal steroidal product of the adrenal gland (1). The 16-keto analogue of this steroid is 5-androsten-3β-ol-16-one, and it will be of much biologic interest to see whether the latter compound has natural occurrence, inasmuch as the 16-ketosteroid, androstan-3β-ol-16-one, is a normal metabolite in the urine of the pregnant mare (2–4).

We have now been successful in preparing 5-androsten-3β-ol-16-one by employing the new synthetic route (5) to 16-ketosteroids recently discovered. In the present synthesis, dehydroepiandrosterone is submitted to nitrosation and zinc-acetic acid reduction by previous methods (6), and the resulting 5-androsten-3β,17β-diol-16-one (I) is esterified with *p*-toluenesulfonyl chloride to produce the 3,17-ditosylate (II). The *p*-toluenesulfonic acid ester at C_3 is then selectively hydrolyzed by refluxing in aqueous acetone containing a small amount of sulfuric acid, and 5-androsten-3β,17β-diol-16-one-17-*p*-toluenesulfonate (III) is thereby produced. This derivative is then submitted to sodium borohydride reduction followed by alkaline cleavage, as previously described (5), to furnish the desired 16-ketosteroid (IV → V).

Our average over-all yield of 5-androsten-3β-ol-16-one, calculated upon dehydroepiandrosterone as starting material, is 56 per cent.

5-Androsten-3β-ol-16-one has no androgenic or myotrophic activity[1] at a level of 3.5 mg. in the bioassay method of Hershberger, Shipley, and Meyer (7). The compound does not inhibit pituitary gonadotropin hypersecretion[1] in parabiotic rats at a total dose of 3.5 mg.

Preparation of 5-Androsten-3β-ol-16-one

Crude 5-androsten-3β,17β-diol-16-one (I) (6), 5.00 gm., was esterified with *p*-toluenesulfonyl chloride at ice bath temperature in our usual

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[1] This bioassay was performed by the Endocrine Laboratories of Madison, Wisconsin, under the direction of Dr. Elva Shipley.

[2] All melting points are uncorrected. Microanalyses and optical rotations are by Dr. E. W. D. Huffman, Denver.
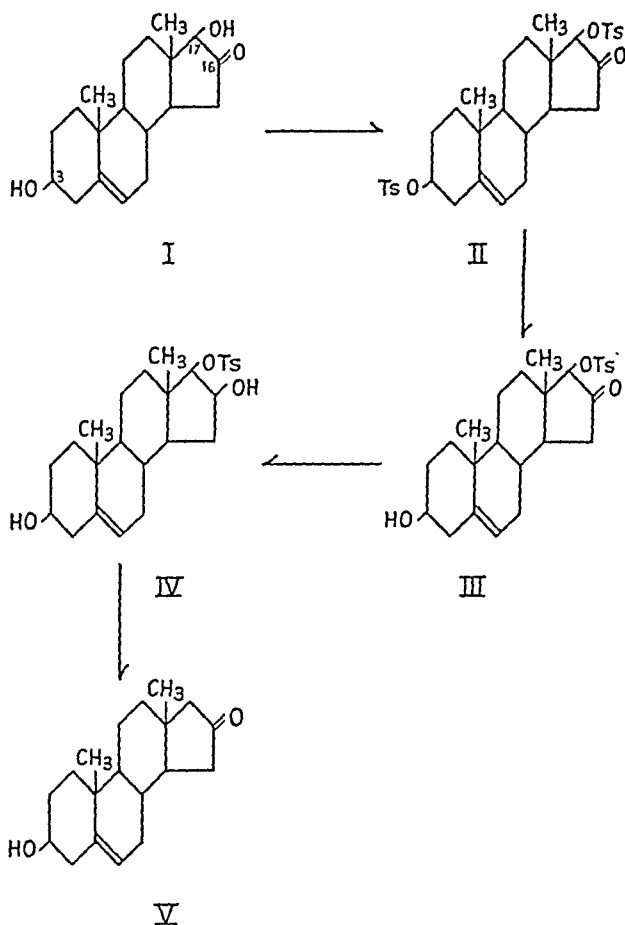
procedure (100 ml. of pyridine; 18 gm. of tosyl chloride) (5).   After 24 hours at room temperature, the ditosylate was precipitated with ice water (2.5 liters), and, after a day at 5°, filtered, washed well with water, and dried in the warm closet (II) (yield, about 10 gm.).

The product (II), as above, was selectively hydrolyzed as follows: To the ditosylate in solution in 625 ml. of alcohol-free acetone were added 225 ml. of water containing 0.52 ml. of concentrated sulfuric acid, and the solution was refluxed for 4 hours, whereupon 225 ml. of water were added and distillation effected to turbidity.   To the hot mixture were added 1100 ml. of 9.1 per cent sodium chloride containing 0.65 gm. of sodium hydroxide.   After a day in the ice box the androstenediolone-17-mono-tosylate (III)[3] was filtered, washed copiously with water, and dried.

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[3] That the 3β-tosyloxy-Δ⁵-steroid (II) is hydrolyzed without inversion at C₃ is borne out by the fact that the final product (V) in the synthesis may be reduced by hydrogen and palladium-charcoal catalyst to furnish authentic androstan-3β-ol-16-one in 80 per cent yield.   Further proof that hydrolysis of the 3β-tosyloxy-Δ⁵-steroid with dilute aqueous acetonic sulfuric acid is accomplished without inversion at C₃ is supplied in Paper XVII of this series.   In Paper XVII it will be shown that such a hydrolysis of 5-androsten-3β,16β-diol ditosylate, followed by epimerization exchange at

The monotosylate (III) was reduced with sodium borohydride in the fashion previously described for the 16-keto-17β-toluenesulfonoxy steroid (5) (125 ml. of methanol and 30 ml. of pyridine; 4.5 gm. of hydride in 50 ml. of methanol). The excess hydride was decomposed with 55 ml. of acetone in 500 ml. of water, and the steroid derivative precipitated with 1.5 liters of 5 per cent sodium chloride containing 25 ml. of concentrated hydrochloric acid.

The well washed androstenetriol-17-tosylate (IV) was dried and dissolved in 900 ml. of boiling 95 per cent ethanol, whereupon 900 ml. of 1 N potassium hydroxide were slowly added to the refluxing solution. After the addition of alkali, the solution was refluxed for 1 hour longer and then distilled to crystallization. The product was recrystallized from acetone-Skellysolve B and from aqueous methanol to yield 3.38 gm. of 5-androsten-3β-ol-16-one, melting at 162–163°. Further recrystallizations raised the melting point to 163.5–165° (balls of fine needles) (V). The crystalline material is hydrated with 0.5 molecule of water, which it has a tendency to regain even after having been fully dried.

$C_{19}H_{28}O_2 \cdot \frac{1}{2}H_2O$. Calculated. C 76.72, H 9.83; loss on drying, 3.03
Found. (a)   " 76.76, " 9.93; "   "   "   2.99
(b)   " 76.79, " 9.86; "   "   "   3.05
$[\alpha]_D^{23.5}$ −242° (c = 0.924 in chloroform as the hemihydrate)

On exposure to the atmosphere, after having been fully dried, the product regained 2.50 per cent weight.

Characterization of 5-Androsten-3β-ol-16-one by Derivatives

5-Androsten-3β-ol-16-one Acetate—Pure 5-androsten-3β-ol-16-one (V), 197 mg., was acetylated in the usual manner with acetic anhydride in pyridine. A recrystallization from aqueous methanol yielded 204 mg., m.p. 125–128°. A further recrystallization sharpened the melting point of the waxy leaves to 127.5–128° (135 mg.).

$C_{21}H_{30}O_3$. Calculated, C 76.33, H 9.15; found, C 76.41, H 9.18

5-Androsten-3β-ol-16-one Benzoate—Pure 5-androsten-3β-ol-16-one (V), 106 mg., was benzoylated at room temperature with benzoyl chloride (1.0 ml.) in pyridine (4.0 ml.). The oil which resulted from precipitation with ice water was recrystallized twice from 95 per cent ethanol to give 100 mg. of broad flat needles melting at 224.5–225.5°.

$C_{26}H_{32}O_3$. Calculated, C 79.55, H 8.23; found, C 79.54, H 8.40

C_{16} plus saponification, yields the same diol as does epimerization exchange plus saponification of 3β-acetoxy-5-androsten-16β-ol tosylate, the final product being 5-androsten-3β,16α-diol in each instance.

5-Androsten-3β-ol-16-one Semicarbazone—Pure 5-androsten-3β-ol-16-one (V), 150 mg., was submitted to semicarbazone formation by refluxing (1.5 hours) in aqueous ethanol (150 mg. of semicarbazide hydrochloride; 210 mg. of crystalline sodium acetate). The semicarbazone was recrystallized twice from benzene and once from aqueous methanol to give 106 mg. of product melting at 232–235°, with decomposition and gas formation. A sample was dried to constant weight *in vacuo* over phosphorus pentoxide at 100° before analysis.

$C_{20}H_{31}O_2N_3$. Calculated, N 12.16; found, N 12.10

Androsten-3β-ol-16-one Oxime—Oximation of pure 5-androsten-3β-ol-16-one (V), 150 mg., was effected in the usual manner by refluxing for 2 hours in aqueous ethanol containing 150 mg. of hydroxylamine hydrochloride and 300 mg. of crystalline sodium acetate. The resulting oxime, in the amount of 150 mg., melted at 197–199.5°, with decomposition. Two further recrystallizations from aqueous methanol raised the melting point to 204.5–205.5°, with decomposition (73 mg.). Before analysis the sample was dried to constant weight *in vacuo* over phosphorus pentoxide at 100°.

$C_{19}H_{29}O_2N$. Calculated, N 4.62; found, N 4.60

In a similar fashion *5-androsten-3β-ol-16-one methoxime* was prepared. It recrystallized from aqueous methanol in small fine needles, m.p. 137.5–138.5°. It was dried to constant weight *in vacuo* at 80° over phosphorus pentoxide before analysis.

$C_{20}H_{31}O_2N$. Calculated, N 4.41; found, N 4.55

Preparation of 3β-Methoxy-5-androsten-16-one

3β-Methoxy-5-androsten-17β-ol-16-one (6) was carried through the steps as given under the "Preparation of 5-androsten-3β-ol-16-one," except that it was not, of course, necessary to go through the reaction involving selective hydrolysis with aqueous acetonic sulfuric acid, the sequence involving only monotosylation at C_{17}, sodium borohydride reduction, and alkaline cleavage. Thus, from dehydroepiandrosterone methyl ether it is possible to prepare pure 3β-methoxy-5-androsten-16-one in 56 per cent over-all yield. From 80 per cent methanol this 16-ketosteroid crystallizes as silky needles and melts at 137.5–138°.

$C_{20}H_{30}O_2$. Calculated, C 79.42, H 10.00; found, C 79.28, H 9.95
$[\alpha]_D^{21}$ −252° (c = 0.932 in chloroform)

SUMMARY

The preparation of 5-androsten-3β-ol-16-one from dehydroepiandrosterone is described.

5-Androsten-3β-ol-16-one was characterized as the acetate, benzoate, methyl ether, semicarbazone, oxime, and O-methyl oxime (methoxime).

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THE TAURINE CONCENTRATION OF ORGANS FROM FED AND FASTED RATS*

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Taurine exists free in the organs of many animals (1–4) and also in combined form, as in the bile salts. The composition of bile salts from over 100 animal species was recently reported, and taurine was the most commonly occurring conjugate (5). The large amount of taurine present in certain tissues seems to be in excess of the amount needed for the formation of bile salts. Moreover, taurine is found in organs of animals such as the rabbit (5), that do not produce taurine-containing bile salts, and it is also formed from inorganic sulfate in the chick embryo (6, 7). From these facts it may be inferred that taurine may play some other rôle besides that of forming bile salts. Considerably more information than that now available is needed to understand the rôle of taurine. In the present work attention has been directed to the taurine concentration of tissues, including the effect of fasting, of starvation, and of the endocrine glands. In addition, the amounts of taurine excreted in the urine have been measured after the administration of cysteine. From the results obtained, it is concluded that taurine is formed even when animals are fasted and that the levels of taurine in most organs do not change to any great extent. Some unexpected sex differences in the taurine concentration of liver were observed in the rat.

EXPERIMENTAL

The determination of taurine was carried out in extracts prepared according to a method described previously (8). The aqueous extracts thus obtained are passed through a 4 × 1 cm. column of Dowex 50-X8 (200 to 400 mesh) in the acid form; 5 cc. of eluate were collected. All of the taurine is found in the eluate, with little contamination from other ninhydrin-reacting substances; when cysteic acid is present, small amounts appear in the eluate. For this reason the eluate is evaporated to dryness in the steam bath and then taken up in 1 cc. of water; 50 μl. of this solution are applied to filter

paper, neutralized with ammonia vapors, and chromatographed with 2,4-lutidine. The extracts of most organs gave a single compact spot which was easily measured quantitatively by any of the known procedures. The elution method with the ninhydrin reagent of Moore and Stein (9) was preferred. This method is reproducible within 10 per cent, and the recovery from the tissues is about 90 per cent. Taurine was determined in measured portions of urine by simply passing the urine through a column of Dowex 50, as described above. The urine was first centrifuged and, if necessary, concentrated to a smaller volume on the steam bath.

Rats of the Sprague-Dawley strain, weighing 200 to 250 gm., were used. The animals were fed Purina chow when not on a special diet. They were sacrificed by decapitation, and samples of the organs to be studied were extracted immediately. Organs from beef, pig, and sheep were obtained from the slaughter-house and put on dry ice within a few minutes after removal.

Hypophysectomized rats were obtained commercially. Gonadectomies and adrenalectomies were performed by the usual procedures. The gonadectomized animals were sacrificed 3 weeks and the adrenalectomized animals 2 weeks after the operation. During this time, they were given salt in their drinking water. A group of gonadectomized male rats received estrogen (estradiol dipropionate, Schering) in doses of 10 γ per day per rat for 10 days. Cysteine was administered by stomach tube. Food was available except in an 8 hour period during which the rats were placed in metabolic cages for the collection of urine.

Results

Taurine Concentration in Organs from Fed Animals—In Tables I and II are recorded the amounts of taurine found in organs from the fed and fasted rat, rabbit, guinea pig, beef, pig, and sheep. Taurine was not found in the liver of the rabbit or guinea pig, even though as little as 0.2 μmole per gm. of tissue could be detected by the method. In nearly all instances more taurine was present in the organs of the rat than in corresponding tissues of the rabbit or guinea pig. It is interesting that in the rabbit the taurine concentration in heart was 15 μmoles per gm., whereas there was no detectable taurine in the liver. As mentioned before, the rabbit produces bile salts containing glycine exclusively (5). Neither taurine nor 2-aminoethanesulfinic acid was detected in the liver of the rabbit after the injection of cysteine (unpublished experiments). In the rat heart, taurine was found in large quantities; indeed, in the male rat, the taurine concentration in the heart was 17 times greater than in the liver. In the female rat, this ratio was much smaller, because the taurine concentration in liver was greater, an unexpected sex difference apparently not controlled ex-

TABLE I

Taurine Content of Rat Organs from Fed and Fasted Rats

Values, in micromoles per gm. of fresh tissue, are averages from six animals, ± the standard error of the mean.

Organ	Male			Female		
	Fed	16 hr. fast	7 day fast	Fed	16 hr. fast	7 day fast
Liver	1.6 ±0.2	2.3 ±0.5	6.1 ±0.3	5.3 ±0.3	3.8 ±0.4	2.3 ±0.3
Kidney	7.4 ±0.6	8.7 ±0.4	10.1 ±0.7	8.1 ±0.8	8.6 ±0.5	9.3 ±0.4
Spleen	11.0 ±0.4	11.6 ±1.0	12.5 ±0.6	13.3 ±0.8	11.9 ±0.5	13.3 ±0.5
Heart	28.4 ±10.5	27.9 ±0.9	30.3 ±1.7	26.7 ±0.6	28.1 ±10.5	26.2 ±1.1
Brain	5.5 ±0.3	6.4 ±0.2	3.0 ±0.6	5.5 ±0.3	5.6 ±0.1	3.5 ±0.3
Small intestine	10.9 ±1.1	10.6 ±2.6	13.3 ±0.9	11.9 ±0.3	10.8 ±10.0	10.3 ±0.5
Muscle	9.4 ±1.1	11.6 ±1.0	13.1 ±1.2	10.7 ±0.8	12.1 ±0.6	11.1 ±0.5
Testis	2.2 ±0.3	2.1 ±0.1	1.8 ±0.1			
Uterus				7.2 ±1.2	6.9 ±1.5	6.8 ±0.5

TABLE II

Taurine Content of Some Organs from Guinea Pig, Rabbit, Pig, Beef, and Sheep

Values in micromoles per gm. of fresh tissue.

Organ	Guinea pig*		Rabbit*		Pig	Beef	Sheep
	Male	Female	Male	Female	Male	Male	Male
Liver..................	0.0	0.0	0.0	0.0	1.1	5.3	3.7
Kidney...............	1.8	1.0	1.9	2.4	7.4	†	4.1
Spleen................	5.6	5.2	5.7	5.6	5.1	3.4	8.8
Heart.................	9.3	12.3	14.6	15.8	33.7	3.5	7.2
Brain.................	1.0	0.6	1.1	1.8	†	†	†
Small intestine.........	3.8	2.3	4.2	2.9	†	†	†
Muscle................	9.1	9.2	1.3	3.7	10.4	8.7	6.8
Testis.................	0.9		0.7		†	†	†
Uterus................		3.5		1.6	†	†	†
Pancreas..............					1.8	1.6	1.4

* Average of values from two animals.
† Not determined.

clusively by sex hormones. Other differences were noted in the taurine content of liver from other species and also in the heart. Relatively low

TABLE III

Endocrine Regulation of Taurine Concentration in Some Organs of Rat

The values, in micromoles per gm. of fresh tissue, are averages from six animals, ± the standard error of the mean.

Organ	Males				Females	
	Hypophy-sectomy	Adrenal-ectomy	Castration	Estrogen	Adrenal-ectomy	Castration
Liver	1.7	1.7	1.6	2.1	2.6	2.7
	±0.3	±0.2	±0.1	±0.4	±0.3	±0.7
Kidney	9.6	6.7	8.2	8.3	6.4	7.4
	±0.3	±0.4	±0.3	±0.4	±0.1	±0.5
Spleen	15.3	11.8	13.8	12.6	11.7	11.9
	±0.6	±0.3	±0.5	±0.4	±0.7	±0.5
Heart	24.3	28.0	27.2	27.1	26.8	25.2
	±0.8	±1.1	±0.9	±1.4	±1.2	±1.2
Muscle	17.0	13.3	11.6	12.7	12.8	10.9
	±0.9	±0.3	±1.3	±0.6	±0.4	±0.9

TABLE IV

Taurine Excretion by Rat

Values in micromoles per 24 hours.

Rat No.	Control period, 24 hrs.	After cysteine,* 24 hrs.
1	47	225
2	72	245
3	69	266
4	53	215
5	57	180
6	76	195
Average..............	62	221

* 100 mg. by stomach tube.

values for the taurine concentration of heart from beef and sheep were found when compared with heart from rodents or pig.

Effect of Fasting—In Table I values for the taurine concentration of organs from rats fasted 16 hours and for 7 days are presented. In both instances the taurine concentration of liver changed significantly and, during the 7 day fast, the increase in liver and in skeletal muscle was marked in male rats. These results are in agreement with those reported by Wu

(10) who found a large increase in the taurine content of both liver and skeletal muscle from male rats after 9 days of fasting. In contrast, the concentration of taurine in the liver of female rats was greatly decreased after a 7 day fast. The other organs changed very little, and in most cases the differences were not statistically significant.

Endocrine Regulation—Sex differences in the taurine content of liver and the contrasting effect of fasting prompted the additional studies listed in Table III. None of the procedures affected the taurine content of the liver of male rats, but hypophysectomy caused an increase in spleen, kidney, and, particularly, in skeletal muscle. The effect of adrenalectomy on the taurine content of skeletal muscle was statistically significant but less marked. Gonadectomy and the treatment of gonadectomized or of intact males with estrogens were ineffective. Adrenalectomy and gonadectomy brought about a significant reduction in the taurine content of the liver of female rats.

Urinary Excretion of Taurine—Table IV presents values for taurine excretion by male rats, calculated on a 24 hour basis. Table IV also contains values for the urinary excretion of taurine after feeding 100 mg. of cysteine by stomach tube. The increase after feeding cysteine was significant but represented only 20 per cent of the total cysteine fed.

DISCUSSION

With few exceptions taurine was found in every organ studied and in concentrations which remained fairly constant, even when the animals were subjected to long periods of fasting. Taurine is excreted almost quantitatively in the urine when administered by mouth or intravenously (11, 12). This apparent stability of taurine in the body was not confirmed by Eldjarn (13) who administered S^{35}-labeled taurine to a human subject and was unable to recover all of the activity in the urine.

Taurine is formed from cystine, cysteine, or indirectly from methionine. The immediate precursor of taurine is 2-aminoethanesulfinic acid (14, 15) which is formed from cysteinesulfinic acid by decarboxylation (16). Hope (17) recently reported data which strongly suggest that cysteic acid decarboxylase could be, in effect, cysteinesulfinic acid decarboxylase. It is of interest that Sloan-Stanley (18) and also Hope (17) found sex differences in the ability of liver to decarboxylate cysteic acid and cysteinesulfinic acid. They reported that the liver of male rats had greater decarboxylating activity than that of female rats, an effect opposite to what might be expected from the results of this study. The amount of taurine observed in organs such as the heart and spleen suggests that these organs concentrate taurine against a high concentration gradient. This is particularly true in the rabbit in which the taurine concentration in the liver is below the limits of

detection by our methods. Portman and Mann (19) recently reported that the urinary excretion of administered taurine-S^{35} is less in rats previously fed a low sulfur diet than in rats fed a high sulfur diet. They also found a high level of S^{35} in tissues 24 hours after the administration of taurine-S^{35}. These levels were higher in the organs of rats fed a diet low in organic sulfur. From these results and ours, it is concluded that taurine plays other rôles in addition to the only one known, namely the formation of bile salts.

SUMMARY

The taurine concentration of several organs from the rat, rabbit, guinea pig, beef, pig, and sheep has been measured. Sex differences were observed in the taurine concentration of the liver of the rat. The effect of fasting, adrenalectomy, hypophysectomy, gonadectomy, and estrogen treatment on the taurine concentration of several organs was studied. Only in a few instances were changes in the concentration of taurine observed. The taurine excretion by the rat was increased after administration of cysteine.

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THE DISTRIBUTION OF FIXED CARBON
IN AMINO ACIDS*

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Experiments previously reported from this laboratory (1) were designed to measure the normal amount of CO_2 carbon which becomes incorporated into the amino acids of the tissues of rats fed a natural diet. This evaluation was made possible by exposing rats to $C^{14}O_2$ for periods long enough to insure a complete exchange of isotope between CO_2 and the organic constituents of several tissues. It was noteworthy that about one-fourth of the carbon of the carboxyl groups of liver aspartic and glutamic acids was derived from carbon dioxide. Smaller amounts of fixed carbon were found in the other non-essential amino acids, and no results were obtained which would suggest metabolic pathways of amino acid synthesis differing from those currently accepted.

The present experiments were designed to measure the extent to which the non-essential amino acids of the diet affect the incorporation of CO_2-derived carbon into tissue amino acids; this was done by offering the animals a diet free of these compounds during exposure to isotopic CO_2 and comparing the results with those previously obtained. We might also expect to learn more about the synthetic pathways in the absence of dilution of synthetic amino acids by dietary amino acids and to measure the maximal extent to which CO_2 may be used as a carbon source. The results obtained show that more CO_2 becomes incorporated into the amino acids when they are absent from the diet, and that the increases differed from one metabolically related group of amino acids to another.

Methods

Exposure of Animals—In previous experiments (1), animals were continuously exposed to $C^{14}O_2$ in closed ventilated chambers for long periods; in the present experiments, however, in order to effect some economy of radioactive material and to simplify operation, one weanling and four adult female Sprague-Dawley rats were exposed to isotopic CO_2 by incorporating $CaC^{14}O_3$ in the diet at a level of 2 μc. per gm. Exposure

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to $C^{14}O_2$ was made continuous by distributing only a limited amount of diet among twenty-four cups placed on a fraction collector moving one station each hour. The amount of food consumed each day was noted and a similar amount, rounded to the next highest gm., was offered for the succeeding day. In any 24 hour period, there were rarely more than two or three cups containing unconsumed ration. Two metabolism cages were placed adjacent to the collection rack, and access to the ration was gained through a short tunnel over the fraction collector.

TABLE I

Composition of Diets

Constituent	Casein	Diet EAA₁	Diet EAA₂	Diet EAA₃	Diet EAA₄
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Sucrose......................	78	81	81	81	80
Corn oil.....................	5	5	5	5	5
Salts........................	4	4	4	4	4
Vitamin mixture..............	0.6	0.6	0.6	0.6	0.6
Casein.......................	12				
L-Histidine·HCl·H₂O..........		0.15	0.15	0.365	0.54
L-Lysine·HCl.................		0.30	0.30	0.375	1.25
DL-Threonine.................		0.30	0.30	0.40	1.0
DL-Isoleucine................		0.60	0.60	0.60	1.0
L-Leucine....................		0.50	0.50	0.30	0.80
DL-Tryptophan................		0.12	0.12	0.20	0.40
DL-Valine....................		0.46	0.46	0.46	1.4
DL-Methionine................		0.30	0.30	0.30	0.60
DL-Phenylalanine.............		0.27	0.27	0.27	0.90
L-Tyrosine...................		0.27	0.27	0.20	
L-Alanine....................		0.50			
Diammonium citrate...........		5.39	6.00	5.9	2.7

That the exposure may be regarded as continuous, even when the animals consumed the contents of the cup almost immediately upon its introduction under the cage, was demonstrated by simulating these conditions in an apparatus designed for the monitoring of the C^{14} radioactivity in the expired air. The level of $C^{14}O_2$ in the expired air of a rat fed once an hour did not vary more than 20 per cent during the hour between feedings.[1]

It was necessary to use a diet which supplied protein only in the form of essential amino acids, yet which was adequate for maintenance of adult rats for long periods. Since no diet described in the literature was adequate for long periods, it was necessary to develop one. Diets EAA₁ and

[1] We wish to thank Mr. Akira Nakao for making these measurements for us.

EAA$_2$, whose composition is shown in Table I, were thought satisfactory until the rat receiving Diet EAA$_1$ began to lose weight prior to the conclusion of the exposure period. In the final experiment, Diet EAA$_3$ was used with apparent success. Subsequently, Womack and coworkers (2) reported a diet whose amino acid composition was similar but somewhat lower. The weanling rat received a level of the essential amino acids as described by Borman *et al.* (3). Arginine was omitted; hence growth was about one-half maximal, as expected. About 10 days were allowed for adjustment to the diet and feeding routine prior to the addition of radioactive $CaCO_3$ to the diet. Urine and feces were collected, and the animals were weighed daily to determine the adequacy of the diet. The length of exposure, diet fed, and weight changes observed are reported in Table II.

Isolation of Amino Acids—After the desired exposure, the rats were killed by exsanguination and dissected, and the organs and muscle were

TABLE II

Length of Exposure and Weight Changes of Rats Fed Various Diets

Diet	Initial weight	Weight change	Exposure period
	gm.	*gm.*	*days*
Casein.............	287	+10	30
EAA$_1$.............	274	−16	31
EAA$_2$.............	306	+15	31
EAA$_3$.............	314	+23	59
EAA$_4$.............	42	+143	122

frozen until analyzed. The tissues were homogenized in 10 per cent trichloroacetic acid, extracted successively with hot 5 per cent trichloroacetic acid and EtOH-ether, and dried. Hydrolysis of the crude protein fraction was accomplished with double distilled HCl in a sealed vessel at 115° overnight. After the excess acid had been removed by vacuum distillation, the amino acid solutions were treated with charcoal and brought to pH 4 to 5 by titration with Dowex 2 in the bicarbonate form.

The amino acids were separated into three fractions on Dowex 2 according to the procedure of Abelson *et al.* (4). The neutral amino acid fraction was concentrated, made 0.2 M in citrate buffer at pH 2.5, and chromatographed at pH 3.42 on Dowex 50 Na$^+$ as described by Moore and Stein (5). The acidic amino acids were adsorbed directly on a 50 cm. column of Dowex 50 H$^+$ and resolved with 1.5 N HCl. The various fractions were concentrated and degraded directly. Arginine was isolated as the flavianate, and free arginine was recovered as described previously (1).

Degradation of Amino Acids—All of the amino acids examined were treated with ninhydrin for measurement of the carboxyl groups (6). The

carbon of the guanidino group of arginine was obtained by hydrolysis with saturated NaOH at 117° (7).

The average radioactivity of the aspartic acid carboxyl groups was obtained with ninhydrin. In earlier work (1), the radioactivity of the β-carboxyl was obtained by transamination of aspartic acid, producing oxalacetate, which was subsequently decarboxylated with Al^{+++}. The results obtained suggested equal labeling of the two carboxyl groups; subsequently, however, the reliability of the method became doubtful. Although not satisfactory in our hands for quantitative results, the method of Ehrensvärd et al. (8), in which chloramine-T is employed for the differentiation of the two carboxyl groups, did suggest some difference between the two groups. Also, frequently the specific activity of the α-carboxyl group of glutamic acid, which is homologous with the β-carboxyl group of aspartic acid, was higher than the value obtained for aspartic acid. These inconsistencies suggested that randomization of the radioactivity in the carboxyl groups was occurring during the preparation of the oxalacetate, owing to other enzyme activities in the crude heart homogenates. Hence a different procedure was tried. A lyophilisate was prepared of Clostridium welchii, grown according to the procedure of Mandeles and coworkers (9), which possessed high aspartic acid β-decarboxylase activity.[2] The specificity of this enzyme was checked by paper chromatography, and 20 mg. samples of radioactive aspartic acid were treated in vacuo at 37° for 1 hour with 90 mg. of lyophilisate in the presence of 10 mg. of sodium pyruvate and acetate buffer at pH 4.9. The specific activities of the β-carboxyl group of aspartic acid which were obtained by this method were larger and were similar to those for the α-carboxyl of glutamic acid; therefore this procedure was adopted.

Glutamic acid was decarboxylated with chloramine-T, and the β-cyanopropionic acid formed was oxidized with concentrated HCl to succinic acid (10) which was then isolated as the silver salt (11). Silver succinate was converted to CO_2 by wet combustion, and the radioactivity was measured, as was that of all other CO_2 samples, by proportional gas counting (12) with an accuracy of ±1 per cent.

The results were calculated as the per cent carbon in each amino acid position derived from the metabolic CO_2 during the exposure period. The assumed isotope level of this CO_2 was based on the specific activity of urea carbon, computed as described previously (13, 14).

RESULTS AND DISCUSSION

The amounts of CO_2-derived carbon found in the amino acids of the rats fed a purified, casein-containing diet and $CaC^{14}O_3$ (Table III) were

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[2] We are indebted to Dr. Mandeles and his coworkers for making the details of their method available to us prior to its publication.

similar to those observed previously in rats fed a natural diet and receiv-
ing the isotopic carbon through the lungs as CO_2 (1). As would be pre-
dicted from previous work (1, 13), the levels of incorporation of fixed car-
bon into the amino acids of liver and intestine of the animals treated for
30 to 31 days were similar to those observed in the adult animal exposed
for 59 days, as well as in the young rat which quadrupled its weight during
the 122 day exposure period. Intestinal muscle protein must differ
markedly from skeletal muscle protein in turnover rate, since the latter

TABLE III

Fixed Carbon in Amino Acids

Exposure	Diet	Per cent fixed carbon in									
		Aspartic acid		Glutamic acid		Proline carboxyl	Arginine		Serine carboxyl	Glycine carboxyl	Alanine carboxyl
		Average carboxyl	β-Carboxyl	α-Carboxyl	Residue		Carboxyl	Guanidine			
						Liver					
days											
30	Casein	18.4	22.8	22.4	0.2	3.8		85	9.4	8.0	5.7
31	EAA₁	31.2		30.8		10.7	8.7	100	11.3	9.2	7.7
31	EAA₂	47		45		11.7	9.3	100	10.2	10.4	10.5
59	EAA₃	35.7	39.8	36.7		14.6	10.8	80 (?)	11.4	11.8	11.7
122	EAA₄	32.0	37.1	29.5	0.1	14.9	14.4	95	10.7	11.1	8.5
						Muscle and intestine					
30	Casein	7.6	10.5	10.2		4.1	4.4	53	6.9	2.8	4.1
31	EAA₁	13.3	14.5	14.9		10.2	7.2	55	9.6	7.8	6.7
31	EAA₂	14.8	17.4	18.0		11.6	8.0	67	10.0	7.6	8.1
59	EAA₃	13.9	16.0	16.4		11.6	10.9	61	11.5	9.4	7.8
122	EAA₄	13.6	15.2	13.7	0.1	13.7	14.1	76	9.8	9.2	6.1

was not in complete isotopic equilibrium even after 59 days. The ob-
served values for skeletal muscle were increased to the values expected at
isotopic equilibrium by use of a factor calculated from the data of Buchanan
and Nakao (15) obtained from measurement of the fixed carbon content
of whole tissues of animals exposed for various periods. The accuracy
of this procedure is evidenced by the similarity of the corrected values and
the uncorrected values obtained from the growing rat in which there can
be no appreciable fraction of the protein which is not in isotopic equi-
librium.

Because intestinal and skeletal muscle protein, although differing greatly
in turnover rate, differed less than 10 per cent in the fixed carbon fraction

of their amino acids, and because an occasional value is missing or was equivocal, the numbers have been averaged and are reported as a single value.

Although some of the amino acids displayed an abundance of fixed carbon, this in no way implies that there is a net gain in carbon through fixation of carbon dioxide in animals as there is in plants. The experiments measure only the obligatory participation of carbon dioxide in the reactions which lead to the formation of a number of compounds. Nor does the increased fixed carbon fraction of a tissue amino acid in its absence from the diet *necessarily* indicate an increased fixation of carbon dioxide, because we may now be observing the normal fixation rate as it is reflected in an amino acid undiluted by a dietary source. There is, however, some correlation between the level of an amino acid in casein, on the one hand, and the increase in fixed carbon fraction of the particular amino acid in the animal fed a diet free of the compound. For example, proline and glutamic acid are plentiful in casein, and, in their dietary absence, their tissue CO_2-derived carbon content increased 2- to 3-fold (Table III). Nevertheless, aspartic acid and arginine, although no more abundant in casein than are serine, glycine, or alanine, showed an increase in their fixed carbon fraction similar to that of glutamic acid and proline, whereas the CO_2 carbon content of the short chain amino acids was only slightly raised by a dietary deficiency (Table III). The slight effect of diet on the incorporation into serine and glycine is in agreement with the conclusions of Arnstein and Neuberger (16), who found that the daily synthetic rate of serine and glycine was 5 to 10 times the usual daily intake of these amino acids and that the rate was essentially independent of their level in the diet. The addition of 0.5 per cent of alanine to Diet EAA_1 had a doubtful effect on the fixed carbon fraction of alanine.

It is also interesting to compare the level of CO_2-derived carbon in the amino acids isolated from liver with those from non-hepatic tissues. Whereas proline and arginine have similar fixed carbon fractions regardless of tissue source, hepatic serine, glycine, and alanine contain about one-third more CO_2 carbon than their counterparts in muscle and intestine. By far the greatest difference between tissues was in the fixed carbon fractions of glutamic and aspartic acids and the guanidino group of arginine. When isolated from the liver, these amino acids contained almost twice as much fixed carbon as those found elsewhere. The lower fraction of fixed carbon in the guanidino group of arginine of the extrahepatic tissues compared to urea cannot be explained simply by relegating all synthesis of urea to the liver. Although these data seem to suggest a lower specific activity for isotopic CO_2 in the non-hepatic tissues, this is unlikely. Buchanan and Nakao (14) examined the fixed carbon content of a number of

bones from all parts of the body and found that the specific activities of new bone carbonate and urea were alike. We have also examined an independent site of CO_2 fixation, the C-6 position of ribonucleic acid adenine and guanine isolated from intestine and liver, and found its specific activity in both tissues to be identical with that of urea.[3]

Examination of the β-carboxyl group of aspartic acid showed that the degree of randomization between the two ends of aspartic acid reaches a value which is quite constant among animals on essential amino acid diets (Table IV). The ratio of β-carboxyl to α-carboxyl was such that, in the rats fed the essential amino acid diets, 6 of 7 malate molecules formed by CO_2 fixation were converted to the symmetrical compound,

TABLE IV

Ratio of Fixed Carbon in α- and β-Carboxyl Groups of Aspartic Acid

Diet	Tissue	$\dfrac{\beta\text{-Carboxyl}}{\alpha\text{-Carboxyl}}$	
Casein	Liver	1.63	
	Intestine	2.22	(1.92)*
EAA$_1$	Muscle	1.38	
EAA$_2$	"	1.38	
EAA$_3$	Liver	1.27	
	Muscle	1.27	
EAA$_4$	Liver	1.38	
	Intestine	1.38	
	Muscle	1.22	(1.33)*

* Average.

fumarate, before conversion to oxalacetate, while fewer than 5 of 7 formed in the casein-fed rat were so randomized.

Upon degradation of the glutamic acid, it was found that over 99 per cent of the fixed carbon in this amino acid was in the α-carboxyl group, in accord with the known reactions of the Krebs cycle (Table III). These results are in contrast, however, to the findings of Hill and Koeppe (17) who report having observed as much as 20 per cent of the total fixed carbon of glutamic acid in the remainder of the molecule. In our rats, at least, it seems clear that there is negligible reversal of the α-ketoglutarate oxidation step. Furthermore, none of the other reactions, which in bacteria (18) or in hen oviduct (19) lead to non-α-carboxyl labeling of glutamic acid from $C^{14}O_2$, appears to occur in our animals.

Glutamic acid is now presumed to be the precursor of the carbon chains of proline and the ornithine moiety of arginine, and it seems to be the

[3] R. W. Swick, D. T. Handa, and A. L. Koch, unpublished data.

sole precursor in the extrahepatic tissues examined. In liver, however, the discrepancy between the fixed carbon fractions of proline and ornithine and of glutamic acid suggests that some other, unlabeled, compound (for example, histidine or lysine) may also serve as a precursor of these two amino acids.

The high level of CO_2 fixation into aspartic (and glutamic) acid of the rat fed Diet EAA_2 deserves some comment. Since the fixed carbon fraction of this amino acid in liver (47 per cent) represents an average of two positions, the real fixed carbon content of aspartic acid is 94 per cent. In other words, there is nearly 1 mole of fixed carbon per mole of dicarboxylic acid in the liver of this animal. Since oxalacetate that has been formed by the reactions of the Krebs cycle contains no fixed carbon, it seems clear that only a very small fraction of the total oxalacetate present was regenerated through the reactions of the Krebs cycle.

SUMMARY

The fixed carbon fraction of the non-essential amino acids of liver, muscle, and small intestine has been measured in the rat, in the presence and absence of a dietary supply of these compounds. The level of incorporation of CO_2-derived carbon is 2 to 3 times as high in proline, arginine, and glutamic and aspartic acids when these amino acids are omitted from the diet. Only a small increase in fixed carbon was observed in serine, glycine, and alanine under these conditions. It was also observed that hepatic proline and arginine seem to have some precursor other than, or in addition to, hepatic glutamic acid, and that the rat is capable of synthesizing much more than catalytic amounts of the dicarboxylic acids from carbon dioxide and triose when necessary.

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CONSTITUTION OF THE HYDROXYSULFAPYRIDINE ISOLATED FROM DOG URINE

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Shortly after the introduction of sulfapyridine into clinical use, Antopol and Robinson (1) described the occurrence of renal lithiasis in experimental animals which received the drug. The uroliths were shown to be composed of N^4-acetylsulfapyridine mixed with smaller amounts of the unchanged drug. Further study of its urinary excretion in the dog disclosed the presence of a hydroxysulfapyridine and a water-soluble hydroxysulfapyridine glucuronide (2).

Weber *et al.* (3) repeated the isolation of the hydroxysulfapyridine from dog urine and demonstrated oxidation of the pyridine ring by hydrolyzing the metabolite to sulfanilic acid and an unidentified aminohydroxypyridine. It was suggested (4) that the hydroxyl group of the hydroxysulfapyridine might be in the 3 position of the pyridine ring. More recently, Bray *et al.* (5) concluded from chromatographic study and color reactions that cleavage of the hydroxysulfapyridine gave an aminohydroxypyridine which was not one of the known aminohydroxypyridines, and by a process of elimination they assigned to the derivative the structure 2-amino-5-hydroxypyridine, although they were unsuccessful in their attempts to synthesize it.

In order to establish more conclusively the structure of the sulfapyridine excretion product, we have now prepared the 2-amino-5-hydroxypyridine by way of a Curtius reaction from 5-hydroxypicolinic acid. The 3-hydroxy- and the 6-hydroxy-2-aminopyridines were also synthesized for comparative purposes. Reaction of the aminohydroxypyridines with 2 equivalents of acetylsulfanilyl chloride yielded the corresponding acetyl-sulfanilamidopyridyl acetylsulfanilates. Alkaline hydrolysis, used to remove the acetyl groups and to cleave the sulfanilic acid ester linkage, liberated the hydroxysulfapyridines. In order to confirm the structure of the metabolite, a sample was isolated from dog urine for comparison with the synthetic products.

EXPERIMENTAL

Methyl 5-Hydroxypicolinate—A solution of 40.5 gm. of 5-hydroxypicolinic acid (6) in 160 gm. of methanol containing 22 gm. of HCl was heated under a reflux overnight and then concentrated to 40 ml. *in vacuo*. The residue was dissolved in 150 ml. of water and treated with $NaHCO_3$. The yield

of crude white solid (m.p. 191–195°)[1] was 33 gm. From the filtrate, 6 gm. of 5-hydroxypicolinic acid were recovered. When recrystallized from methanol, the product melted at 193.5–195.5°.

$C_7H_7O_3N$. Calculated, N 9.2; found, N 9.0, 9.1

5-Hydroxypicolinic Acid Hydrazide—To 32 gm. of methyl 5-hydroxypicolinate was added a solution of 30 ml. of $NH_2NH_2 \cdot H_2O$ in 75 ml. of water. The solution was warmed and allowed to crystallize. After neutralizing the solution, 32.5 gm. of white crystals were filtered off. When recrystallized from water, the 5-hydroxypicolinic acid hydrazide melted at 270° (decomposition).

$C_6H_7O_2N_3$. Calculated, N 27.4; found, N 27.8

2-Amino-5-hydroxypyridine Hydrochloride—A solution of 8 gm. of 5-hydroxypicolinic acid hydrazide in 100 ml. of ethanol was treated with 5 gm. of dry HCl. At room temperature, a single portion of 5.5 gm. of *n*-butyl nitrite was added, followed by sufficient additional nitrite to produce a positive test for nitrous acid. The mixture was warmed until the solid dissolved and was then quickly chilled. Addition of 100 ml. of ether served to hasten crystallization of 9.3 gm. of the azide hydrochloride. This material exploded at 154°. It was suspended in 100 ml. of toluene and refluxed until gas evolution stopped. The dark green isocyanate was separated and transferred to 50 ml. of 20 per cent HCl. After being refluxed until no more gas was given off, the solution was concentrated to dryness *in vacuo*. Solution of the residue in ethanol, treatment with charcoal, and precipitation with ether yielded 5 gm. of crude product (m.p. 100–110°). Several crystallizations from ethanol-ether gave the pale yellow crystalline 2-amino-5-hydroxypyridine hydrochloride which melted at 124–126°. (Weber *et al.* (3) reported a melting point of 126° for the compound obtained by hydrolyzing the sulfapyridine metabolite.)

$C_5H_6ON_2 \cdot HCl$. Calculated. C 41.0, H 4.8, N 19.1
                         Found.   " 41.0, " 4.7, " 19.2, 19.0

A purple color formed with ferric chloride and the indophenol test was positive.

2-Amino-3-hydroxypyridine Hydrochloride—This compound, m.p. 194–197°, was prepared according to Bray *et al.* (5).

$C_5H_6ON_2 \cdot HCl$. Calculated, N 19.1; found, N 18.9, 18.6

2-Amino-6-hydroxypyridine Sulfate—This compound, m.p. 228° (decomposition), was prepared according to Titov and Levin (7).

$C_5H_6ON_2 \cdot \frac{1}{2}H_2SO_4$. Calculated, N 17.6; found, N 17.8, 17.8

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[1] All melting points are corrected.

2-Amino-3-pyridyl Acetylsulfanilate—To 10 gm. of 2-amino-3-hydroxy-pyridine hydrochloride in 20 ml. of pyridine were added 16.5 gm. of acetyl-sulfanilyl chloride. After the mixture had cooled, 400 ml. of water were slowly added. The product, 17.9 gm., was soluble in dilute HCl but insoluble in NaOH. The white compound, from ethanol, melted at 187.5–189°.

$C_{13}H_{13}O_4N_3S$.   Calculated, N 13.7; found, N 13.4, 13.5

2-Acetylsulfanilamido-3-pyridyl Acetylsulfanilate—A solution of 17.8 gm. of crude 2-amino-3-pyridyl acetylsulfanilate in 35 ml. of pyridine was treated with 15 gm. of acetylsulfanilyl chloride at 60° for 10 minutes. The cooled mixture was diluted with 300 ml. of water to give 24.2 gm. of hydrated material. The product was dissolved in NaOH solution, treated with charcoal, and reprecipitated. The white crystalline material melted at 259°.

$C_{21}H_{20}O_7N_4S_2$.   Calculated, N 11.1; found, N 11.2, 11.3

2-Acetylsulfanilamido-5-pyridyl Acetylsulfanilate—Acetylsulfanilyl chlo-ride (45 gm.) was added to a solution of 14.5 gm. of crude 2-amino-5-hydroxypyridine hydrochloride in 75 ml. of pyridine. The temperature of the solution was maintained at 65° for 10 minutes. The solution was cooled and poured into a mixture of 100 ml. of HCl and 400 ml. of water. The solid was separated, reprecipitated from NaOH with acetic acid, and then crystallized from ethanol. The gray powder (10.5 gm.) melted at 242°. Recrystallization from ethanol gave a white powder which melted at 244–246°.

$C_{21}H_{20}O_7N_4S_2$.   Calculated, N 11.1; found, N 11.0

2-Acetylsulfanilamido-6-pyridyl Acetylsulfanilate—This compound was prepared from 2-amino-6-hydroxypyridine sulfate according to Phillips (8). The product was not purified, but was used directly in the hydrolytic procedure.

3'-, 5'-, and 6'-Hydroxysulfapyridines—The acetylsulfanilamidopyridyl acetylsulfanilates were hydrolyzed in boiling 20 per cent NaOH for 2 hours. The 3'-hydroxysulfapyridine, recrystallized from 50 per cent acetic acid containing a trace of ethylenediaminetetraacetic acid to remove a green color, melted at 207–209° and had a pK_a of 7.6.

$C_{11}H_{11}O_3N_3S$.   Calculated.   C 49.8, H 4.2, N 15.8
                        Found.   " 50.0, " 4.1, " 15.7, 15.9

The 6'-hydroxysulfapyridine melted at 234–235°. Phillips (8) reported a melting point of 239–240°.

$C_{11}H_{11}O_3N_3S$.   Calculated, N 15.8; found, N 15.8, 15.8

The 5'-hydroxysulfapyridine crystallized from water as a white hemihydrate which dehydrated slowly above 120°.

$C_{11}H_{11}O_3N_3S \cdot \frac{1}{2}H_2O$. Calculated. C 48.2, H 4.4, N 15.3, H_2O 3.3
Found.   " 48.4, " 4.4, " 15.4, " 3.7

When dried *in vacuo* over phosphorus pentoxide at 100°, the product melted at 191.5–192.5°. The pK_a was 7.5.

$C_{11}H_{11}O_3N_3S$. Calculated, C 49.8, H 4.2, N 15.8; found, C 49.8, H 4.1, N 15.6

The product gave positive ferric chloride and diazo tests and a positive indophenol reaction. The ultraviolet absorption spectra at pH 7.0 of
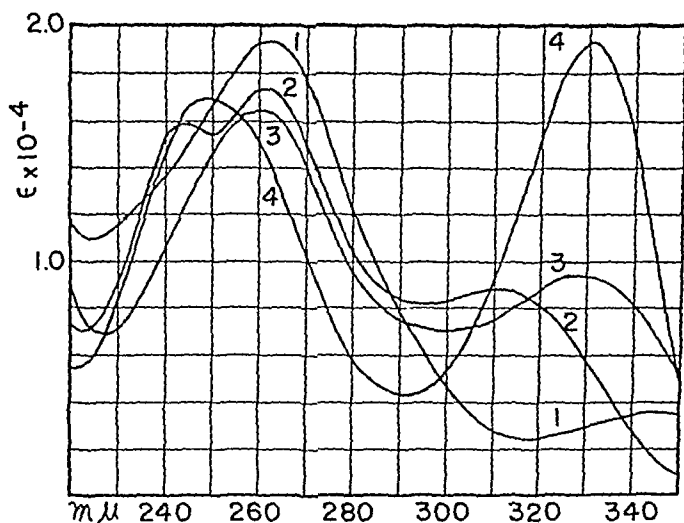
FIG. 1. Ultraviolet absorption of 5'-hydroxysulfapyridine (Curve 1), sulfapyridine (Curve 2), 3'-hydroxysulfapyridine (Curve 3), and 6'-hydroxysulfapyridine (Curve 4) at pH 7.0.

sulfapyridine and the three hydroxysulfapyridines are shown in Fig. 1. Introduction of the hydroxyl groups most markedly influences the absorption in the near ultraviolet. These curves suggest that the 5'-hydroxysulfapyridine is the metabolite, but quantitative agreement with the curve published earlier (1) is not good. By varying the pH, a family of curves was obtained with 5'-hydroxysulfapyridine (Fig. 2). The curve recorded at pH 4.0 is in satisfactory agreement with the earlier one, although the fine detail at the maximum could not be demonstrated with the simpler Beckman apparatus used in the present study. The isosbestic points common to Curves 1, 2, and 3 at 2760 A and 3100 A indicate, we think, anionic dissociation of the sulfonamide which has a pK_a of 7.5. Curves 4 and 5 do not pass through these points because in these more acidic solutions a third molecular species, presumably a cation, is formed. At pH 12 (not shown in Fig. 2) the maximum is shifted to 2520 A and the

$\epsilon \times 10^{-4}$ is increased to 2.58 as a result of dissociation of the phenolic group.

Isolation of Metabolite from Dog Urine—Urine,[2] obtained from a dog which received 3.5 gm. of sulfapyridine per day, was filtered, acidified with acetic acid, and extracted three times with equal volumes of butanol. The butanol extracts were discarded and the urine was treated with lead acetate to maximal precipitation. The precipitate was discarded, and the filtrate was made alkaline. The basic lead precipitate was suspended in water and the lead was removed as the sulfide. The filtrate was concentrated and refluxed in the presence of 10 per cent NaOH for 5 hours. The
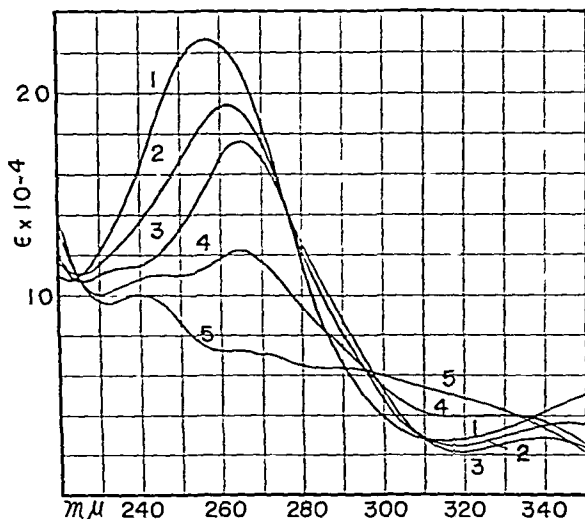
Fig. 2. Ultraviolet absorption of 5′-hydroxysulfapyridine at varying pH values. Curves 1 to 5 were taken at pH 9, 7, 4, 2, and 1, respectively.

cooled solution was acidified, and the precipitated hydroxysulfapyridine was recrystallized from 50 per cent alcohol and from water. The product was isolated as white hydrated crystals which dehydrated slowly above 120°. When dried *in vacuo* over phosphorus pentoxide the melting point was 191.5–192.5°. Weber *et al.* reported a melting point of 190°.

$C_{11}H_{11}O_3N_3S$. Calculated, C 49.8, H 4.2, N 15.8; found, C 49.7, H 4.2, N 15.6

As reported earlier (4), the product gave a positive indophenol reaction when the test was performed as described (9). The blue color remained

[2] Limited study of a urine sample by means of paper chromatography indicated the presence of unchanged sulfapyridine, small amounts of 5′-hydroxysulfapyridine, and large amounts of a water-soluble conjugate. Chromatographically, the conjugate appeared to be a mixture, but in our hands hydrolysis has liberated only 5′-hydroxysulfapyridine.

in the aqueous phase. In view of this test, which is generally assumed to be given only by phenols unsubstituted in the para position, and in analogy with the excretion of 3′-hydroxysulfaquinoxaline (10), a tentative structure, 3′-hydroxysulfapyridine, was suggested (4) for the sulfapyridine metabolite. It is now demonstrated that the metabolite is, in fact, the 5′ isomer. The melting point of the metabolite was not depressed when it was mixed with synthetic 5′-hydroxysulfapyridine. The ultraviolet absorption spectra of the metabolite and the synthetic product at pH 7.0 were identical (Fig. 3).
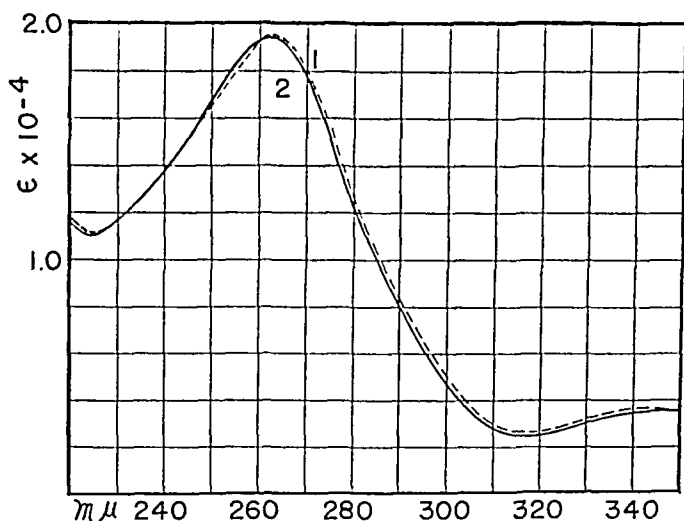
FIG. 3. The ultraviolet absorption of 5′-hydroxysulfapyridine in a phosphate buffer at pH 7.0. Curve 1, product isolated from dog urine; Curve 2, synthetic product.

SUMMARY

2-Amino-5-hydroxypyridine has been prepared by way of a Curtius reaction from 5-hydroxypicolinic acid. This derivative was converted to the acetylsulfanilamidopyridyl acetylsulfanilate, which was in turn hydrolyzed to yield 5′-hydroxysulfapyridine. The 3′- and the 6′-hydroxysulfapyridines were similarly prepared. Comparison of the physical and chemical properties of these synthetic products with the metabolite isolated from the urine of dogs fed sulfapyridine established the structure of the metabolite as 5′-hydroxysulfapyridine.

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THE EFFECT OF COBALT ON HEME SYNTHESIS BY BONE MARROW IN VITRO*

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Since the demonstration by Waltner and Waltner (1) that cobalt will produce a polycythemia in rats, this effect has been produced in various other laboratory animals and human beings. However, the mechanism by which cobalt produces erythroid hyperplasia of the bone marrow remains obscure. This unique action of cobalt continues to be of interest because of its importance in leading to a better understanding of erythropoiesis and because of the possible therapeutic rôle of cobalt in various anemias.

There have been a number of attempts to explain cobalt polycythemia in terms of an anoxic mechanism (2). Barron and Barron (3) observed an inhibition of the respiration of immature red cells by small amounts of cobalt. They concluded that this inhibition led to premature release of young red cells into the circulation and hence to polycythemia. However, Warren et al. (4) were not able to confirm these findings. They observed that cobalt in concentrations of 10^{-4} to 10^{-2} M did not interfere with the respiration of bone marrow or reticulocytes *in vitro* unless the concentration was as high as 0.01 M.

Attempts have been made to link cobalt with the cytochrome system of biological oxidation. Schultze (5) noted a close relationship between the cytochrome oxidase activity of the bone marrow and the ability to form hemoglobin. Levey (6) gave intravenous injections of cytochrome c to cobalt-polycythemic rats, but failed to abolish the polycythemia. However, it was pointed out that cytochrome c administered intravenously does not appear in tissue cells.

Although the hypothesis that cobalt produces cellular anoxia is an attractive one, the experimental evidence does not justify such an assumption. Attempts to explain cobalt polycythemia in terms of cellular bone marrow anoxia are made difficult by the fact that anoxia itself has not been shown to stimulate bone marrow directly (7).

The development of a method for measuring hemoglobin synthesis by bone marrow *in vitro* by measuring the rate of incorporation of radio-

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active glycine into heme has provided a new technique for studying the mechanism of action of cobalt (8). The rate of heme synthesis is only one part of the process of erythropoiesis, but it is a specific function of erythroid cells. This approach is more indicative of erythropoiesis than studies based on oxygen measurements alone, as it has been noted that heme synthesis and oxygen consumption are not always parallel (8). In this investigation the effect of cobalt on bone marrow *in vitro* was studied by the simultaneous measurement of heme synthesis and oxygen consumption.

Method

Rabbits weighing 4 to 5 pounds were used. The technique for preparing the marrow, incubating it with radioactive glycine, and determining the radioactivity of the hemin has already been described (8).

Cobaltous chloride[1] was dissolved in an aliquot of bicarbonate-free serum, and progressive dilutions were made with the same serum to give the final molarity desired. The pH of the serum was adjusted to 7.35 to 7.40 just before use. Each experiment lasted 10 hours, and the oxygen consumption was measured for the first 3 hours.

The glycine-2-C^{14} had a specific activity of 0.2 mc. per mmole and a final concentration of 1.4×10^{-3} M. In the experiment with α-C^{14}-acetate the specific activity was 0.02 mc. per mmole, and the final concentration was 12.5×10^{-3} M.

Oxygen consumption and hemin radioactivity were determined in duplicate and averaged. The results were expressed as the per cent of the control without cobalt.

Results

Five experiments were carried out on normal bone marrows which contained 26 to 52 per cent erythroid cells (Fig. 1). There was no effect of cobalt on heme synthesis until concentrations of approximately 10^{-4} M were reached, at which point inhibition began and was complete at 10^{-2} M. There was no significant effect of cobalt on oxygen consumption at concentrations up to 10^{-2} M. At no cobalt concentration was there a significant stimulation of oxygen consumption or heme synthesis.

Cobalt is known to form complexes with many substances, including amino acids (9). The observed inhibition of heme synthesis by cobalt could be an artifact produced by complex formation between cobalt and the radioactive glycine. Acetate, as well as glycine, is a heme precursor (10), but does not form complexes with cobalt. Therefore, two experiments with radioactive acetate rather than glycine were carried out.

[1] Courtesy of Lloyd Brothers, Inc., Cincinnati 3, Ohio.

The results obtained with acetate were very similar to those in which glycine was used (Fig. 1).

The dissociation of oxygen consumption and heme synthesis produced by cobalt could be due to a differential effect on the erythroid as compared to the myeloid cells. Three experiments were performed on marrows containing 70 to 78 per cent erythroid cells obtained from rabbits given acetylphenylhydrazine. The results still showed a dissociation of oxygen consumption and heme synthesis essentially identical with those
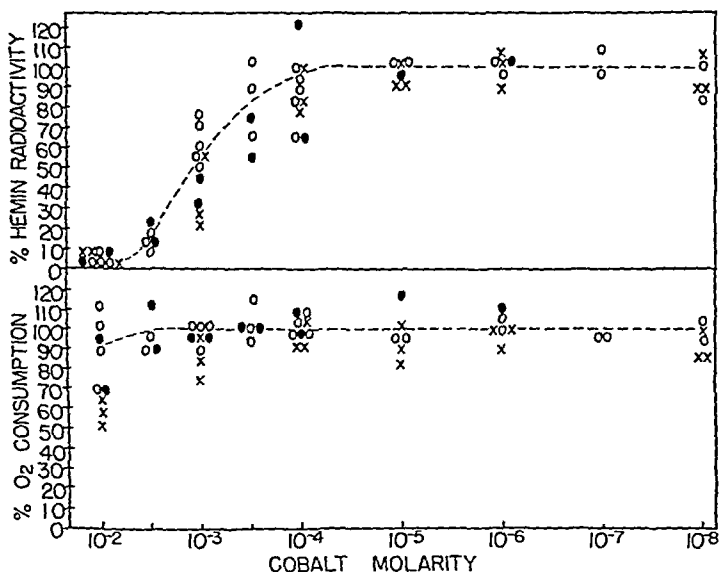
FIG. 1. The data are expressed as per cent of the control serum without cobalt. O, normal marrow with glycine-2-C^{14}; ●, normal marrow with α-C^{14}-acetate; ×, erythroid marrow with glycine-2-C^{14}. The curve is drawn through the data for the normal marrow with glycine-2-C^{14}.

of the normal marrow (Fig. 1), except at a cobalt concentration of 10^{-2} M, at which there was perhaps a greater inhibition of oxygen consumption.

DISCUSSION

It has been reported that the normal level of cobalt in human serum is approximately 10^{-9} M (11). The serum level of cobalt in cobalt polycythemia has not been measured with accuracy, but is probably 10^{-5} M or less (12). If cobalt stimulates bone marrow directly, a zone of stimulation should be found between 10^{-5} and 10^{-9} M. Our experiments in the range from 10^{-2} to 10^{-8} M failed to show stimulation of heme synthesis or oxygen consumption. Such a failure suggests that cobalt does not have a direct effect on the bone marrow. However, these experiments do not

exclude this possibility. It may be that cobalt stimulates some phase of erythropoiesis other than heme synthesis. For instance, stimulation of cell proliferation would not be detected in experiments of 10 hours duration, such as these. It may be that cobalt *in vivo* is converted to a biologically active complex which acts directly on the bone marrow.

The most important observation here is that cobalt at concentrations greater than 10^{-4} M inhibits heme synthesis, but does not interfere with oxygen consumption until concentrations of 10^{-2} M are reached. The fact that cobalt "poisons" hemoglobin synthesis at concentrations well below those which inhibit oxygen consumption would seem to exclude the possibility that cobalt can cause polycythemia by a mechanism of bone marrow anoxia.

SUMMARY

The effect of cobalt *in vitro* on bone marrow was studied by measuring oxygen consumption and heme synthesis simultaneously.

Cobalt in concentrations of 10^{-2} to 10^{-8} M did not stimulate either oxygen consumption or heme synthesis.

Cobalt at concentrations greater than 10^{-4} M inhibits heme synthesis, but does not interfere with oxygen consumption until concentrations of 10^{-2} M are reached. This is interpreted to mean that cobalt does not produce polycythemia by a mechanism of bone marrow anoxia.

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METABOLISM OF PYRUVIC ACID-2-C14 AND HYDROXYPYRUVIC ACID-2-C14 IN ALGAE*

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The metabolism of pyruvic acid in the light and dark by *Scenedesmus* has been examined with two aims: to study the relationship of its metabolism to that of the intermediates of $C^{14}O_2$ fixation and to compare its metabolic fate with that of hydroxypyruvate.

The metabolism of hydroxypyruvic acid was compared with that of $C^{14}O_2$ fixation in order to ascertain whether it might be a photosynthetic intermediate or a substrate for the enzyme systems present. Since phosphohydroxypyruvate is a possible carboxylation product of ribulose diphosphate, the metabolism of the free acid was considered of interest. Stafford *et al.* (1) observed high D-glyceric acid dehydrogenase activity in a variety of leaves and suggested the possibility of its function in pentose phosphate carboxylation. The present work suggests rather that hydroxypyruvic acid may act largely as a substrate for transketolase or a hydroxypyruvic acid oxidase (2).

Pyruvate-2-C^{14} and hydroxypyruvate-2-C^{14} were prepared on a small scale. The green alga, *Scenedesmus*, was allowed to photosynthesize in the presence of these substrates at low pH consistent with optimal assimilation of relatively strong organic acids. The products formed in the algae during light and dark periods were examined by two-dimensional paper chromatography.

EXPERIMENTAL

Pyruvic Acid-2-C^{14}—Carbonyl-labeled pyruvic acid was synthesized according to the method of Anker (3). Sodium acetate was converted to acetyl bromide by heating over benzoyl bromide (4). The acetyl bromide was distilled *in vacuo* and converted to acetyl cyanide upon standing at room temperature over cuprous cyanide. An ethereal solution of acetyl cyanide saturated with hydrogen chloride was hydrolyzed during 30 min-

utes at 0° by addition of the theoretical amount of water to crystalline pyruvamide. This was hydrolyzed to pyruvic acid which was stored as the crystalline sodium salt. Its specific activity was 17.5 μc. per mg.; the theoretical value, based upon the barium carbonate used, was 18.2 μc. per mg.

Chromatography of Pyruvic Acid-2-C^{14}—The labeled pyruvate gave one major spot containing 98 per cent of the radioactivity with R_F 0.59 in phenol and R_F 0.43 in butanol-propionic acid solvent. The minor spot with R_F 0.25 × 0.35 accounted for no more than 1 per cent of the total activity.

Light Metabolism of Pyruvate-2-C^{14} by Scenedesmus—1 day-old *Scenedesmus* was suspended in fresh nutrient (10 per cent suspension) and acidified to pH 3.5 with dilute HCl. After 15 minutes of photosynthesis in air, 1.0 mg. (17.5 μc. per mg.) of pyruvic acid per 100 mg. of cells was added and the air stream was continued for the duration of the fixations until the algae were rapidly filtered (3 seconds) through Celite and killed with hot 80 per cent ethanol. The solid remaining was extracted with 100 per cent and 20 per cent ethanol. The extracts were combined and concentrated. In a 45 minute light experiment at pH 3.5, 13 per cent (660,000 c.p.m.) of the pyruvate was fixed in the soluble fraction (Fig. 1), while in a 40 minute dark fixation (Fig. 2) 7 per cent was fixed in this fraction. It would appear that CO_2 fixation in the light diminished the total fixation of pyruvic acid and diluted the labeled reservoirs of intermediates.

The concentrated extracts were chromatographed two-dimensionally and radioautographs were prepared. The products observed in the radioautographs of several 35 to 45 minute light fixation experiments were the same. The lipides contained a major fraction of the radioactivity; glutamate had over 2 times as much as any other single product. Other products included aspartate, malate, glutamine, succinate, phosphate esters involved in hexose synthesis, sucrose, alanine, serine, glycine, and citrate with radioactivity decreasing in that order. No appreciable amount of glycolic acid appeared. Sucrose was hydrolyzed and the products were cochromatographed with carrier fructose and glucose. Although the 45 minute light fixation included some pyruvate, its presence was apparently due to incomplete washing of the cells.

A marked difference was observed in the lipide and phospholipide areas from the light and dark fixations. *3 times as much lipide were formed in the light* (3200 c.p.m., 35 minutes) as in the dark (1100 c.p.m., 40 minutes), although glutamic acid was labeled to the same extent in both cases. The phospholipide areas from chromatograms of both experiments had approximately equal radioactivities (1500 c.p.m. dark, 1600 c.p.m. light).

A more hydrophilic group of substances associated chromatographically with the sulfolipides (lower R_F than phospholipides) had one-fourth as
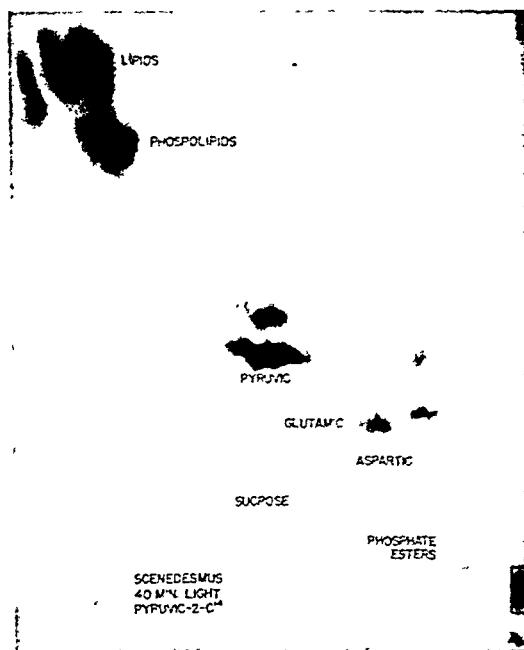
FIG. 1. Products of light metabolism of pyruvic acid-2-C14. The chromatogram was developed to the left in phenol and upwards in butanol-propionic acid.
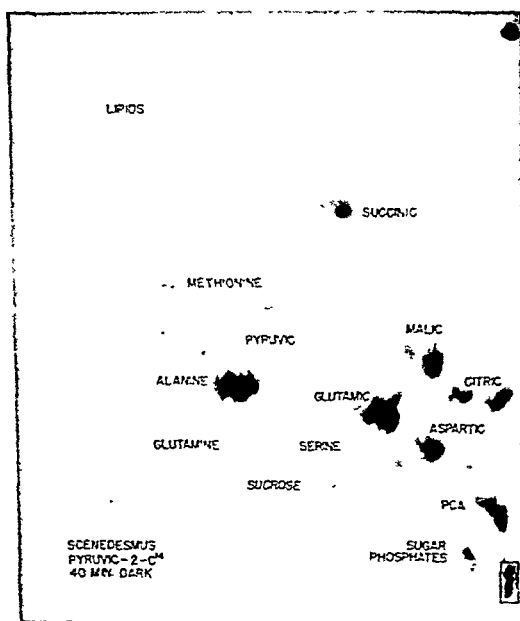
FIG. 2. Products of dark metabolism of pyruvic acid-2-C14. PGA = phosphoglyceric acid.

much activity as the fats and was not significantly different with illumination.

It is apparent from a comparison of these radiographs with those of normal $C^{14}O_2$-labeled photosynthesis intermediates that sucrose and its phosphorylated precursors have a much lower specific activity than do glutamate and aspartate. This would infer a dilution of the sugar intermediates by non-labeled CO_2 and polysaccharide reservoirs. In the light, such dilution does not seriously affect incorporation of pyruvate-2-C^{14} into tricarboxylic acid cycle intermediates.

Dark Metabolism of Pyruvate-2-C^{14}—Dark metabolism of pyruvate-2-C^{14} differs markedly from that of $C^{14}O_2$. The products (Fig. 2) include many of those characteristic of $C^{14}O_2$ photosynthesis, in addition to those respiration intermediates which become labeled by exchange in dark $C^{14}O_2$ fixations (5).

The products seen in the radiographs included sucrose and hexose phosphates in quantities exceeding those formed with $C^{14}O_2$ in the dark. Sucrose was identified by hydrolysis and cochromatography. Monophosphates of galactose, mannose, glucose, and fructose were identified by phosphatase hydrolysis and cochromatography of the free hexoses. The products differed strikingly from those of photosynthesis in that neither sedoheptulose monophosphate nor ribulose diphosphate was observed. Uridine diphosphate glucose was observed and contained the major amounts of labile glucose phosphate. Lipides were observed but not in the considerable amounts formed in the light.

Bromopyruvic Acid-2-C^{14}—The preparation, described by Sprinson and Chargaff (6), was adapted to small scale reactions. Pyruvic acid is converted to the corresponding bromopyruvic acid and the bromopyruvic acid is hydrolyzed just before use to the hydroxypyruvic acid. To 130 mg. of pyruvic acid at 50°, 244 mg. of liquid bromine were added. As the reaction starts, the bromine disappears and hydrobromic acid is formed. The mixture was placed in a vacuum desiccator over moist sodium hydroxide pellets and the solvent was removed by suction. Bromopyruvic acid crystallized and 190 mg. were recovered after drying (equivalent to 77 per cent). The bromopyruvic acid had a specific activity of 10.5 ± 1 μc. per mg. compared to the calculated 11.5 μc. per mg. The dilution may be due to the formation of some dibromopyruvic acid.

Paper Chromatography of Bromopyruvic Acid—Crystalline bromopyruvic acid gave a single spot when run in phenol (R_F 0.49) and butanol-propionic acid (R_F 0.65) on Whatman No. 4 paper. When made alkaline, hydroxypyruvate appeared at once. In the presence of sodium ion, bromopyruvate solutions were observed to form double spots upon phenol development on Whatman No. 1 paper.

Chromatographic Properties[1] *of Hydroxypyruvic Acid-2-C*[14]—Bromopyruvic acid was hydrolyzed in dilute alkali as done by Sprinson or in phosphate buffer when small quantities of radioactive material were involved. Three major labeled products resulted when bromopyruvic acid was hydrolyzed in mild alkali. When the bromo acid was hydrolyzed with 0.1 N sodium hydroxide by slow addition to maintain pH 7 to 8, the radiograph had four spots with R_F values in phenol and butanol-propionic acid as follows: for Spot A, 0.15 × 0.35; Spot B, 0.31 × 0.38; Spot C, 0.31 × 0.64; and Spot D, 0.44 × 0.64. Spots A and B (hydroxypyruvate) contained over 80 per cent of the C^{14}. From the slight streaking observed in the second (butanol-propionic acid) direction and the identity of Spot C (by cochromatography) with bromopyruvate, it may be concluded that residual bromopyruvate had been hydrolyzed upon drying the phenol or upon acidic action by propionic acid to give a good yield of hydroxypyruvate.

Hydrolysis in phosphate buffer at pH 6.0 gave hydroxypyruvate and glycolate with very little residual bromopyruvate. The phenol R_F values, of course, were changed somewhat with the pH. Spot D has been identified as glycolic acid. This apparently arises from decarboxylation of tartronic acid semialdehyde followed by air oxidation.

Both bromopyruvate and hydroxypyruvate give single spots after treatment of the solution with Dowex 50 cation resin to remove sodium ion. No evidence for separation of tautomers (dihydroxyacrylic acid and tartronic acid semialdehyde) during chromatography was observed. The major radioactive components are shown in Fig. 3.

Photosynthesis with Hydroxypyruvic Acid-2-C[14] *by Scenedesmus*—Previous experience with glycolic and formic acid feeding experiments in this laboratory suggested that a low pH would favor assimilation of the labeled substrate. A 3 hour and a 40 minute photosynthesis with dark fixation controls was performed with *Scenedesmus* at pH 3.5. The radioactivity in the soluble products was one-third to one-fourth of that fixed in the corresponding experiments with pyruvate-2-C[14]. The presence of residual bromopyruvate in the substrate suggested a possible inhibitory effect analogous to that of iodoacetate. Hence, $C^{14}O_2$ fixations in dilute phosphoric acid (pH 3) were performed with or without hydroxypyruvate prepared by identical hydrolysis conditions from bromopyruvate. No significant effect upon products of 1 minute $C^{14}O_2$ fixation by the possible bromopyruvate contaminant was observed.

The hydroxypyruvate-2-C[14] assimilations in the light in the presence of CO_2-free air gave low yields of labeled products. These included gluta-

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[1] The authors are indebted to Mr. R. A. Sherrer for an examination of the chromatographic properties of hydroxypyruvic acid.

mate, aspartate, lipides, and sucrose. In a 60 minute light experiment, however an unusual amount of glycolic acid was observed, in addition to abnormally high glycine concentrations and lesser amounts of serine, ala-

GLYCOLIC
ACID

BROMOPYRUVIC
ACID

HYDROXYPYRUVIC ACID

BROMOPYRUVATE
HYDROLYSATE
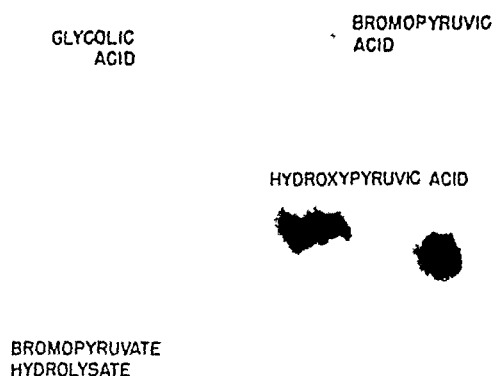
Fig. 3. Hydrolysis products of bromopyruvic acid-2-C¹⁴

GLYCOLIC

ALANINE   GLYCINE   HYDROXY
                                           PYRUVATE

SERINE

SUCROSE

SCENEDESMUS
HYDROXYPYRUVATE-2-C¹⁴
60 MIN LIGHT, AIR
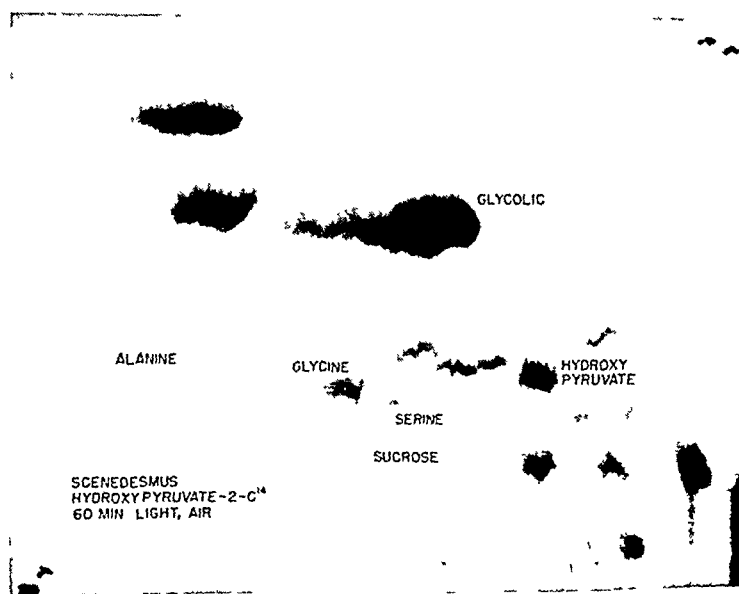
Fig. 4. Products of light metabolism of hydroxypyruvic acid-2-C¹⁴

nine, glutamic acid, and phosphate esters (Fig. 4). No glyceric acid was observed.

Degradation of Glycolic Acid from Products of 40 Minute Light Assimilation of Hydroxypyruvate-2-C¹⁴ in CO₂-Free Air—Glycolate eluted from fresh chromatograms was degraded according to the method of Schou *et al.* (7).[2] The carboxyl carbon was found to have 89 per cent and the α-carbon 18 per cent of the total C^{14} in the starting acid.

DISCUSSION

The copious yield (~85 per cent) of carboxyl-labeled glycolate from hydroxypyruvate-2-C¹⁴ is in accord with decarboxylation by transketolase or hydroxypyruvic acid oxidase (8, 2), followed by oxidation of the glycolyl derivative to free glycolic acid, while the minor yield of α-labeled glycolate

$$HOCH_2-C^*O-COOH \xrightarrow{-CO_2} \xrightarrow{-[2H]} HOCH_2-C^*OOH$$
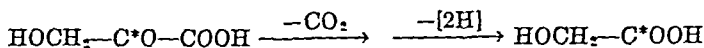
(~15 per cent) can be accounted for by the non-enzymatic decarboxylation which is observed with preparations of hydroxypyruvate. Any reas-

$$HOCH_2-C^*O-COOH \rightleftharpoons OCH-C^*HOH-COOH \rightarrow$$

$$CO_2 + OCH-C^*H_2OH \xrightarrow{oxidation} HOOC-C^*H_2OH$$
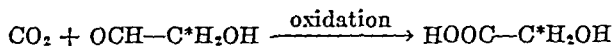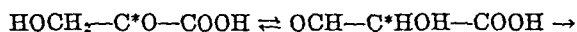
similation of $C^{14}O_2$ from the oxidized substrate would have resulted in symmetrically labeled glycolate (7). Glycolic acid is readily oxidized further in *Scenedesmus* and hence can serve as a respiratory intermediate.

The rather slow observed formation of carbohydrates from hydroxypyruvate suggested that its aerobic metabolism by algae is largely oxidative even though enzyme systems for its incorporation are present. Pyruvate-2-C¹⁴ is converted to lipides and carbohydrates at least 10 times faster than is hydroxypyruvate under comparable conditions. It seems unlikely, then, that hydroxypyruvate is a normal intermediate or a source of glycolyl groups involved in carbohydrate synthesis.

The primary effect of light on pyruvate-2-C¹⁴ metabolism is on the rate of lipide synthesis. The rate of tricarboxylic acid cycle oxidation of pyruvate-2-C¹⁴ is not completely inhibited in the light. This suggests an independent tricarboxylic acid cycle metabolism for exogenous pyruvate which proceeds at a similar rate in dark and light, while lipide formation is light-dependent. This appears contrary to previous observations of light inhibition of respiration of photosynthetic intermediates (9, 10), which was attributed to maintenance of thioctic acid in the reduced form incapable of pyruvate oxidation. However, since a fraction of the thioctic

[2] We are indebted to the kindness of Dr. E. M. Thain for the chemical degradation.

acid of the cell is not associated with the chloroplasts, it is likely that a separation of oxidative sites allows extrachloroplastic pyruvate oxidation to proceed in the light as well as in the dark. The equality of light and dark respiration, observed by Brown (11) for organisms with highly organized chloroplasts, apparently demands such an interpretation.

SUMMARY

1. Pyruvic and hydroxypyruvic acids are metabolized by *Scenedesmus*.

2. The products of metabolism of pyruvic-2-C[14] and hydroxypyruvic-2-C[14] acids are essentially identical to those of C[11]O$_2$ fixations.

3. Lipides are rapidly formed in the light from both substrates. In the dark the major products are tricarboxylic acid cycle intermediates.

4. It does not appear likely that free hydroxypyruvic acid is a photosynthetic intermediate.

5. Tricarboxylic acid cycle intermediates are formed from exogenous pyruvate as fast in the light as in the dark.

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THE GROWTH RESPONSE OF MAMMALIAN CELLS IN TISSUE CULTURE TO L-GLUTAMINE AND L-GLUTAMIC ACID

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PLATE 1

Twelve L-amino acids (arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine) have been found essential for the growth of two mammalian cell lines in tissue culture, a mouse fibroblast[1] (strain L) (1) and a human uterine carcinoma cell (strain HeLa) (2). The minimal vitamin requirement of these cell lines has also been defined (3).

As will be shown here, under the conditions of the present experiments, glutamine proved similarly essential for the survival and growth of both cell lines. In a medium containing the twelve amino acids previously shown to be essential, the seven demonstrably essential vitamins, glucose, electrolytes, and serum protein, both the mouse fibroblast and the human carcinoma cell degenerated and died unless the medium was supplemented with glutamine. The quantitative aspects of this glutamine requirement, and the limited degree to which it could be satisfied by glutamic acid, are described.

Methods and Materials

The methods of maintaining stock cultures of the two cell lines, of setting up replicate cultures with the various experimental media, and of cell enumeration to evaluate the growth response have been described in previous papers dealing with the amino acid and vitamin requirements of the mouse fibroblast (1, 3) and the HeLa carcinoma cell (2, 3). A helpful modification in the cell-counting procedure has been the use of disodium Versenate (disodium salt of ethylenediaminetetraacetic acid) to disperse the cells, instead of mechanically scraping them from the surface of the culture flask. The flask was drained, and 3 ml. of fresh medium at pH

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[1] This cell was originally cultivated from normal connective tissue, but tissue cultures have occasionally produced sarcomata, particularly after irradiation of the recipient mice (personal communication from K. K. Sanford and W. R. Earle).

7.0, which contained Versenate at 0.005 M, were added. Within 5 to 10 minutes the cell sheet had come off the glass. The cells were then dispersed by pipetting back and forth and stained for hemocytometer enu-

TABLE I

Basal Medium for Cultivation of HeLa Cell and Mouse Fibroblast

L-Amino acids*		Vitamins†		Miscellaneous	
	mM		*gm. per ml.*		*per cent*
Arginine	0.1	Biotin	10^{-6}	Glucose	0.1‡
Cystine	0.05 (0.02)§	Choline	10^{-6}	Penicillin	0.005¶
Glutamine‖	2.0 (1.0)§	Folic acid	10^{-6}	Streptomycin	0.005¶
Histidine	0.05 (0.02)§	Nicotinamide	10^{-6}	Phenol red	0.0005¶
Isoleucine	0.2	Pantothenic acid	10^{-6}	Dialyzed horse	(1)§
Leucine	0.2 (0.1)§	Pyridoxal	10^{-6}	serum	
Lysine	0.2 (0.1)§	Thiamine	10^{-6}	Dialyzed hu-	5
Methionine	0.05	Riboflavin	10^{-7}	man serum	
Phenylala-	0.1 (0.05)§				
nine		Salts‡			
Threonine	0.2 (0.1)§				
Tryptophan	0.02 (0.01)§		*per cent*		
Tyrosine	0.1	NaCl	0.68		
Valine	0.2 (0.1)§	KCl	0.04		
		$NaH_2PO_4 \cdot H_2O$	0.014		
		$NaHCO_3$	0.22		
		$CaCl_2$	0.02		
		$MgCl_2$	0.008		

* Conveniently stored in the refrigerator as a single stock solution containing 20 times the indicated concentration of each amino acid.

† Conveniently stored frozen as a single stock solution containing 100 or 1000 times the indicated concentration of each vitamin.

‡ Conveniently stored in the refrigerator in two stock solutions, one containing NaCl, KCl, NaH_2PO_4, $NaHCO_3$, and glucose at 10 times the indicated concentration of each and the second containing $CaCl_2$ and $MgCl_2$ at 20 times the indicated concentration.

§ The figures in parentheses are for mouse fibroblast.

¶ Conveniently stored frozen as a single stock solution containing 100 times the indicated concentrations of penicillin, streptomycin, and phenol red.

‖ Conveniently stored frozen as a 100 mM stock solution which is added to the medium in appropriate concentration at the time of feeding.

meration by the addition of an equal volume of the citric acid-crystal violet stain of Sanford *et al.* (4).

EXPERIMENTAL

Essentiality of Glutamine for Growth of Mouse Fibroblast and Human Carcinoma Cell—The basal media used for th̶ ̶ ̶g̶r̶o̶w̶t̶h̶ ̶of the two cell lines

are shown in Table I. When the culture flasks (1) were inoculated with 100,000 to 200,000 cells and when, 24 hours later, after the cells had adhered to the glass, the overlying fluid was replaced with a glutamine-deficient medium, the cells died within a few days. However, if glutamine were added to the medium, and if fresh glutamine were then added each time the culture fluid was changed, the cells rapidly grew out to form a solid sheet on the surface of the glass. The growth in the presence of the
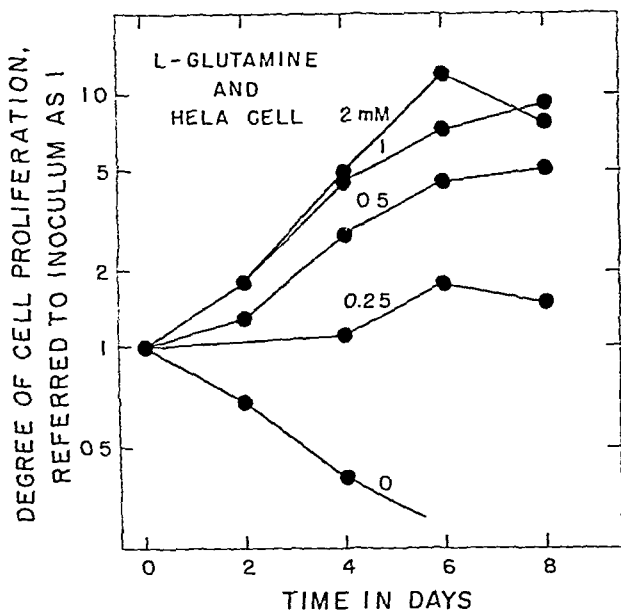
TEXT-FIG. 1. The rate of growth of the HeLa cell at varying concentrations of L-glutamine.

glutamine and the early death of the cultures in its absence are illustrated for the HeLa cell in Figs. 1 to 3 and for the mouse fibroblast in Figs. 4 to 7.

Ineffectiveness of D-*Glutamine*—With the twelve essential amino acids, only the L isomers had been found active; the D-amino acids were wholly inactive, but even a large excess did not inhibit the growth response to the L isomer. Similarly, D-glutamine had no growth-promoting effect on either cell line. The courtesy of Dr. Alton Meister and Dr. Leon Levintow in providing this compound for study is gratefully acknowledged.

Quantitative Glutamine Requirement—Glutamine in various concentrations was added to the medium of Table I at the time of the first feeding. At each subsequent feeding (every 2 days in the case of the mouse fibroblast and daily in the case of the HeLa cell) freshly prepared glutamine solutions were added in appropriate concentration to the culture fluids.

Typical growth response curves are shown in the top sections of Tables II and III. The concentration which permitted maximal growth of the HeLa cell under the conditions of the present experiments was 1 to 2 mM, while

TABLE II

Growth Response* of Human Carcinoma Cell (HeLa) to Glutamine and to Glutamic Acid

Supplement to medium of Table I	Concentration of L-glutamine or L-glutamic acid, mM										
	40	30	20	10	5	2	1	0.5	0.2	0.1	0
	Growth response in 6 days†										
L-Glutamine				6.5		8.1	5.7		1.1	0.6	0.1
					9.7	8.5		5.6	2.9	2.2	
						10		3.9		0.2	
				10.3		11.7		4.6		1.2	
						11.8	7.3	4.6	1.8		
			7.6		8.1		10.6		2.1		0.03
L-Glutamic acid	0.4		2.4	1.2	0.5	0.3					0.2
			2.9								
			4.1	0.6							
	7.7	9.1	8.0	0.5							0.2
	4.8	8.6	9.5	6.5							0.6
L-Glutamic acid + NH₄Cl (1 mM)			7.7								
			8.9								
				4.4		0.3					0.2
			10.7	9.6							0.9
L-Glutamic acid + NH₄Cl (1 mM) + ATP (2 γ per ml.)			8.0								
			8.1								
			6.5	4.2	0.4						0.2

* The basal medium of Table I is employed, supplemented as indicated.

† Referred to inoculum as 1. Each row represents a different experiment, and each number is the average of three replicate flasks.

a somewhat lower concentration (0.2 to 0.5 mM) sufficed for the optimal growth of the mouse fibroblast. The rate of growth of the HaLa cell at varying concentrations of glutamine is presented in Text-fig. 1, which shows the maximal response obtained at a concentration of 1 to 2 mM.

L-*Glutamic Acid As Substitute for Glutamine*—With the HeLa cell, L-glutamic acid could be substituted for glutamine, but the optimal concentration was 20 mM, approximately 10 to 20 times the maximally effective

concentration of glutamine.   As shown in Table II, even this large excess of glutamic acid was usually not as effective as glutamine.   The relatively slow growth response to glutamic acid was largely due to an initial lag (*cf.* Text-fig. 2).   When multiplication began, however, it was at the same rate as that obtained from the outset with glutamine.   On the addition of NH$_4$Cl in the optimal concentration of 0.5 to 1 mM, the growth response to glutamic acid was usually, but not invariably, improved.   The rate of growth then approached that observed at the optimal concentration of glutamine.   The addition of ATP (adenosine triphosphate) at 1 to 2 γ per ml. had no significant effect.
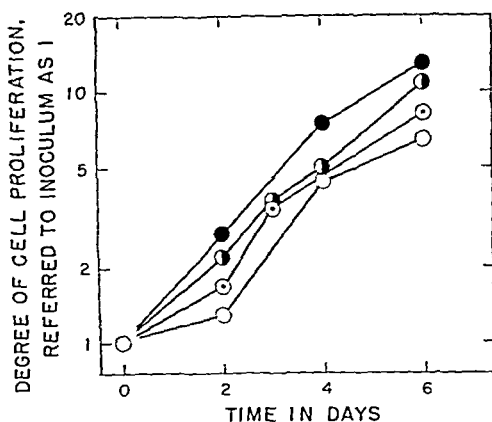
TEXT-FIG. 2. The rate of growth of the HeLa cell at optimal levels of L-glutamine and L-glutamic acid (with and without NH$_4$Cl and ATP).   O, glutamic acid (20 mM); ⊙, glutamic acid + NH$_4$Cl (1 mM) + ATP (2 γ per ml.); ◑, glutamine (2 mM); ●, glutamine + glutamic acid + NH$_4$Cl + ATP.

However, quite different results were obtained with the mouse fibroblast.   When this cell was grown in a medium containing only twelve essential amino acids but no glutamine, glutamic acid alone in any concentration in the range 0.1 to 40 mM failed to substitute for glutamine (lower portion of Table III).   The cells did not multiply and after 6 days showed degenerative changes.   On the addition of NH$_4$Cl in the optimal concentration of 1 mM, a slight growth-promoting effect was occasionally observed (*cf.* Table III).   The addition of ATP at concentrations of 0.5 to 2 γ per ml. again had no effect, and higher concentrations were toxic.

It is of interest that Fischer (5) had found that the addition of approximately 1.5 mM glutamine to a medium consisting of dialyzed serum, dialyzed embryo extract, dialyzed plasma, Tyrode's solution, glycine, and cystine greatly increased the area of growth around a culture explant.

Glutamic acid at the same concentration was, however, inactive and actually caused a slight retardation of growth.

Effect of Full Complement of Amino Acids on Growth Response to L-Glutamine and on Growth-Promoting Activity of L-Glutamic Acid—The previously cited experiments were carried out in a medium in which the cells

TABLE III

Growth Response* of Mouse Fibroblast (Strain L) to Glutamine and Absence of Comparable Response to Glutamic Acid, NH_4Cl, and ATP

| Supplement to medium of Table I	Concentration of L-glutamine or L-glutamic acid, mM									
	40	20	10	5	2–3	1	0.5	0.2–0.25	0.1	0
	Growth response in 6–7 days†									
L-Glutamine			4		4.6	6.5	4.5	4.4	2.8	0.16
				3.6	6.1	8	7.5	3.9	2.5	0.7
					8.3	11.8	10.8	7.3	3.7	0.91
L-Glutamic acid			0.3		0.69		0.62	0.45		0.28
			0.3	0.4	0.5	0.5	0.5			0.5
	0.27	0.3	0.49	0.6	0.46					0.4
	0.25	0.8	1.1	1.5		1.0	1.0		0.7	0.5
L-Glutamic acid + NH_4Cl (1 mM)					0.9					0.5
					0.9					0.5
	0.43	0.36	0.91	0.96	1.29					0.71
					2.2	1.7				0.5
	0.025	1.1	1.5	2.3	3.3					0.75
L-Glutamic acid + NH_4Cl (1 mM) + ATP (2 γ per ml.)				0.1	0.9					0.6
					0.9					0.5

* The basal medium of Table I is employed, supplemented as indicated.

† Referred to inoculum as 1. Each row represents a different experiment, and each number is the average of three replicate flasks.

were under the necessity of synthesizing alanine, aspartic acid, glycine, hydroxyproline, proline, and serine. It became of interest to determine whether an adequate supply of these amino acids would affect the quantitative requirement for glutamine or the growth-promoting activity of glutamic acid.

With both the HeLa cell and the mouse fibroblast, the addition of a mixture of these amino acids to the medium, each at a concentration of 0.2 mM, had a glutamine-sparing action. As shown in Table IV, the ED_{50}

level of glutamine was thereby reduced to approximately one-half its normal level. There was still, however, an absolute requirement for glutamine, and the further addition of glutamic acid, NH_4^+, and ATP still failed to permit significant growth of the mouse fibroblast in a glutamine-free medium.

Failure of α-Ketoglutaric Acid, Asparagine, Ornithine, or Proline to Substitute for Glutamine—Asparagine or ornithine, at concentrations of 20, 2, or 0.2 mM, failed to substitute for glutamine; both the HeLa and L cells died in media containing these compounds in lieu of glutamine. Proline and α-ketoglutaric acid were similarly inactive at these concentrations

TABLE IV

Illustrating Glutamine-Sparing Action of Non-Essential Amino Acids for HeLa Cell and Mouse Fibroblast

Cell strain	Concentration of 6 non-essential amino acids*	Concentration of L-glutamine, mM								ED$_{50}$ of glutamine‡
		2	1	0.5	0.2	0.1	0.05	0.02	0	
		Growth response in 5–6 days†								
	mM									mM
HeLa	0	8.5		5.6	2.9	2.2	1.2		0.2	0.37
	0.05	11.1	9.0	8.6	4.4	2.4	1.0		0.2	0.30
	0.2	12.2	11.0	9.3	6.0	4.3	2.4		0.2	0.20
Mouse fibroblast	0		7.8	7.1	3.04	2.1	1.1	0.62	0.5	0.28
	0.2			6.6	6.0	3.1	1.8	1.4	0.7	0.11

* Alanine, aspartic acid, glycine, hydroxyproline, proline, serine, each at the concentrations indicated.

† Referred to inoculum as 1.

‡ Approximate concentration permitting 50 per cent of maximal growth.

whether tested alone or in combination with 0.5 to 1 mM NH_4Cl and ATP at 0.5 to 2 γ per ml. As stated in the preceding section, the addition of a full complement of the non-essential amino acids often caused a significantly enhanced growth response to glutamine. Even under these circumstances, however, neither ornithine nor proline substituted for glutamine.

DISCUSSION

It was not surprising that glutamine should prove essential for the survival and growth of mammalian cells in a medium containing only twelve essential amino acids, glucose, protein, and the necessary vitamin supplement. It was, however, not anticipated that glutamic acid would be only one-tenth to one-twentieth as active as glutamine in the case of the HeLa

cell and usually devoid of activity in the case of the L cell. The simplest explanation is that these two cells are relatively impermeable to glutamic acid. However, as will be discussed in a following paper, in media containing as little as 0.01 to 0.1 μmole per ml. of C^{14}-labeled glutamic acid, the latter was actively incorporated into cell protein by both types of cells, whether in the presence or absence of glutamine, and the concentration of free glutamic acid in the cell was regularly much greater than that in the surrounding fluid.

An alternative explanation which is consistent with all the data reported here is that, at least under the conditions of the present experiments, and in conformity with the findings of McIlwain *et al.* (6) with *Streptococcus pyogenes*, glutamine plays a vital rôle in the cellular economy which glutamic acid as such cannot perform. Given a high concentration of glutamic acid (20 mM), the HeLa cell can apparently synthesize enough glutamine to provide for its needs, presumably by the reaction described by Krebs (7), Elliott (8), Speck (9), and others.

$$\text{Glutamate} + NH_3 + ATP \rightarrow \text{glutamine} + \text{adenosine diphosphate} + \text{phosphate}$$

It is significant in this connection that, in the present experiments, the growth response of the HeLa cell to glutamic acid was usually slightly, but definitely, increased by the addition of NH_4^+. In the mouse fibroblast, however, this synthesis is apparently insufficient to supply the minimal needs of the cell for glutamine. Glutamic acid in any concentration could substitute for glutamine only irregularly, and at best incompletely (*cf.* Table III), and even the addition of a full complement of amino acids did not affect the specific requirement for glutamine. Neither cell line required glutamic acid over and above glutamine, either because glutamine is readily converted to glutamic acid or because the cell can synthesize the latter amino acid as readily as it can, *e.g.*, glycine, serine, alanine, or proline.

Asparagine did not substitute for glutamine, either in the L or HeLa cell. Further, although large concentrations of glutamic acid could substitute for glutamine in the case of the HeLa cell, neither α-ketoglutaric acid, proline, nor ornithine was similarly effective, with or without added NH_4^+ and ATP. If the HeLa cell can make glutamic acid from these compounds, as described for mice by Stetten (10), this synthesis is quantitatively insufficient to provide for survival and growth.

The present experiments provide no clue to the nature of the metabolic reaction (or reactions) mediated by glutamine and not by glutamic acid. Meister and Tice (11) and Meister, Sober, Tice, and Fraser (12) showed that there were a number of keto acids, both natural and unnatural, with which glutamine transaminated actively, while glutamic acid had no significant activity. Other workers (13, 14) have similarly found glutamine

to be more effective than glutamic acid in several specific transamination reactions, as well as in a non-enzymatic transamination of glyoxylate (15). Under the conditions of the present experiments, glutamine may therefore provide an essential transaminating mechanism.   The fact that the glutamine requirement was significantly reduced by the addition to the medium of six non-essential amino acids is consistent with this thesis.   More recently, glutamine has been found (16–18) to be an intermediate in purine synthesis, the amide N appearing as nitrogens 3 and 9 of the hypoxanthine ring (18).   Since, under the conditions of the present experiments, the cells were under the necessity of synthesizing their total purine and pyrimidine requirements, this and similar reactions may help account for the specific need for glutamine.   Glutamine has also been reported to be involved in the biosynthesis of glucosamine phosphate (19, 20).   Finally, glutamine as such may be required for incorporation into cell proteins.

SUMMARY

Under the conditions of the present experiments, both a mouse fibroblast (strain L) and a human carcinoma cell (strain HeLa) required L-glutamine for survival and growth in tissue culture.   The maximally effective concentration was 0.2 to 0.5 mM for the fibroblast, and 1 to 2 mM for the carcinoma cell.   The addition of a full complement of amino acids had a glutamine-sparing action, but did not affect the glutamine requirement qualitatively.

Glutamic acid at the optimal concentration of 20 mM substituted for glutamine in the case of the HeLa cell, but not with the mouse fibroblast. The further addition of NH4Cl often increased the growth response of the former cell and occasionally permitted slight growth of the latter.

Arginine, ornithine, α-ketoglutaric acid, and proline were inactive as substitutes for glutamine.

It is suggested that glutamine is an essential metabolite under the conditions of the present experiments, and that the growth-promoting activity of high concentrations of glutamic acid for the HeLa cell reflects its conversion to glutamine.   The possible nature of the metabolic reaction (or reactions) uniquely mediated by glutamine is discussed in the text.

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EXPLANATION OF PLATE 1

Figs. 1 to 3. The 6 day growth response of the HeLa cell to varying concentrations of L-glutamine (in the basal medium of Table I). Magnification, 100 ×.

Figs. 4 to 7. The 6 day growth response of the mouse fibroblast to varying concentrations of L-glutamine (in the basal medium of Table I). Magnification, 110 ×.
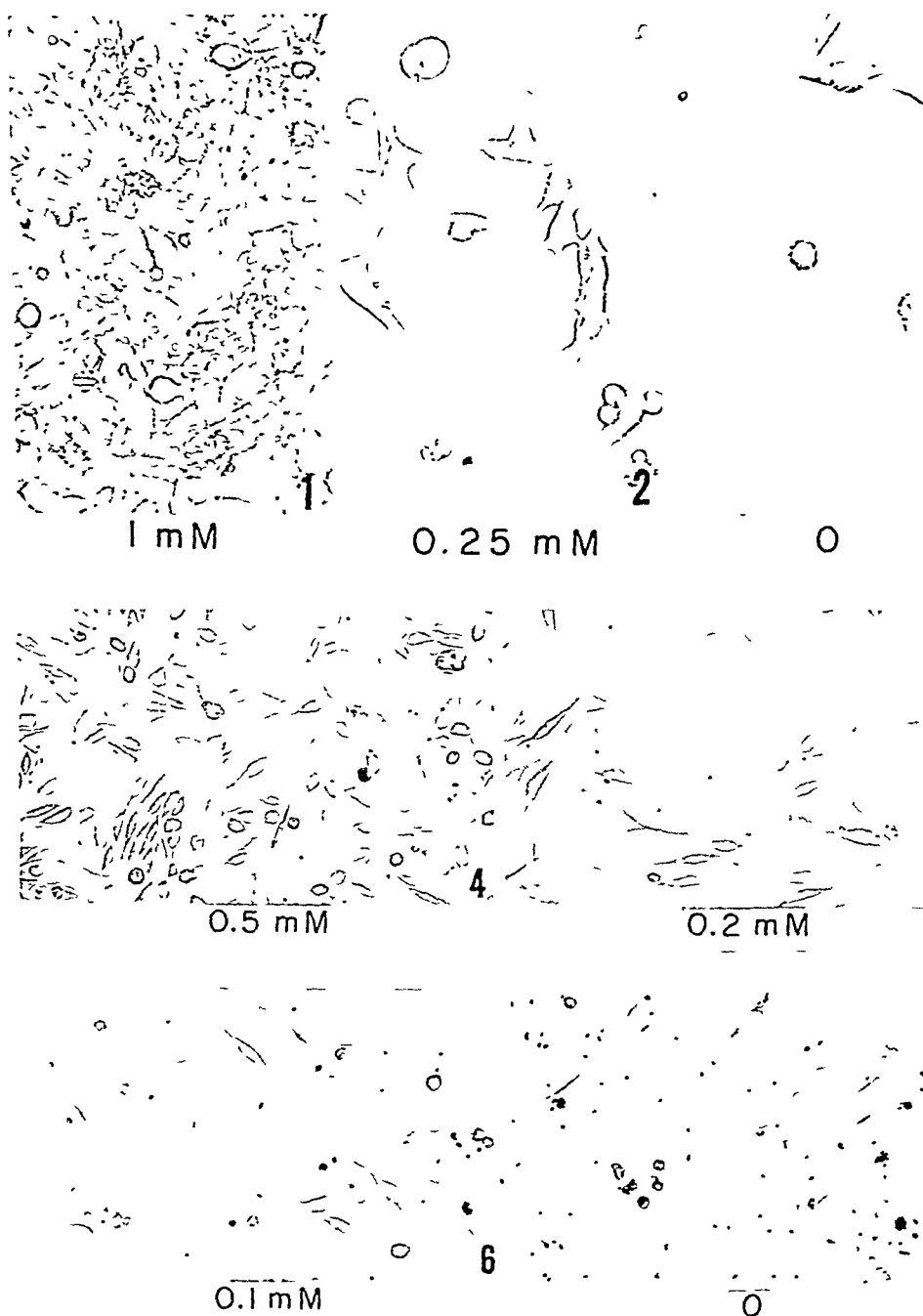
1

I mM

0.25 mM   O

4

0.5 mM   0.2 mM

6

0.1 mM

O

COFACTOR REQUIREMENTS FOR ENZYMATIC DENITRIFICATION

I. NITRITE REDUCTASE*

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The reduction of nitrate by plants and microorganisms leads either to ammonia or to gaseous nitrogen as the final product. Although some light has been shed on the mechanism of conversion to ammonia by cell-free extracts or partially purified enzyme preparations, little attention has been given to the enzymatic denitrification of nitrite to nitrogen gas. It has recently been shown that reduction of nitrite to nitrogen by cell-free extracts of *Pseudomonas stutzeri* and *Bacillus subtilis* is accompanied by the production of small amounts of nitric oxide and nitrous oxide (1).

It has since been possible to show that enzyme preparations from *P. stutzeri* can convert nitrite to nitric oxide and the latter to nitrogen gas. Both of these steps require copper and iron as well as pyridine and flavin nucleotides for full activity.

This paper describes the purification, properties, and the cofactor requirements of the over-all system $(NO_2^- \rightarrow NO)$.

Materials and Methods

Enzyme Preparation—It was reported earlier that active extracts were obtained from cells of *P. stutzeri* grown at 25° or below (1). However, more uniformly active extracts were obtained from the cells grown for 18 to 24 hours at 37° in a medium containing 1 per cent trypticase (Baltimore Biological Laboratory), 0.5 to 1.0 gm. per cent of KNO_3 (or 0.3 per cent KNO_3 and 0.2 per cent $NaNO_2$), and 0.1 per cent Difco yeast extract.

12 liters of an 18 to 24 hour culture were centrifuged in the Sharples centrifuge at room temperature. Towards the end of the centrifugation, about 2 liters of cold water were passed through the centrifuge. The packed cell mass at this stage still contained small amounts of nitrate or nitrite which disappeared during handling or purification. The harvested cells were mixed with 3 times their weight of alumina powder (No. 1557

AB, Buehler, Ltd., Chicago) and either ground immediately at 4° or stored in the deep freeze at −18° without any loss of activity for at least 4 weeks. The ground cells were then extracted three times with an equal volume of ice water, and the extract was centrifuged for 30 to 40 minutes at about 20,000 × g to eliminate cell débris and the small particles containing the electron carrier system (1). The sediment was washed three times with cold water. The total volume of the combined extracts and the washings was about 6 times the wet weight. All subsequent manipulations were carried out at 4–6°.

To the extract thus prepared solid ammonium sulfate was added to 0.4 saturation. The precipitate formed was separated by centrifugation and dissolved in 20 ml. of water. By further addition of ammonium sulfate, 0.55 and 0.70 fractions were prepared in a similar manner. Very little precipitate was obtained between 0.7 and full saturation. All fractions were then dialyzed for 5 to 18 hours against several changes of cold distilled water (1–3).

Methods of Assay—The utilization of nitrite was measured by the diazo reaction (4). For the determination of gas evolution the usual Warburg technique was used. The main compartment contained the enzymes and buffer. One side arm contained nitrite and the hydrogen donor substrate. The other contained cofactors. Unless stated otherwise, the following conditions were employed: phosphate buffer, pH 6.8, 0.08 M final concentration; temperature 30°; final volume of the mixture 2.4 to 3.0 ml.; nitrogen in the gas phase. 0.2 ml. of 20 per cent KOH was placed in the center well. The reaction was started at zero time by tipping in the substrates. Control samples as indicated were run simultaneously.

The hydrogen donor substrates used were either boiled yeast extract, *l*-malate, succinate, glucose-6-phosphate, or *d*-isocitrate, as the case may be. The total gas formed represented nitrogen and nitric oxide (1–3). It was possible to absorb completely the nitric oxide evolved during the reaction by the use of alkaline sulfite placed in one side arm. Consequently, nitrogen was the only product appearing in the gas phase.

Furthermore, the simultaneous measurement of the total volume of gas formed and the amount of nitrite which had disappeared at a particular time afforded a means of obtaining exact values of NO and N_2 formed in the gas phase by solving the two following simultaneous equations in which all terms are expressed in microliters under standard conditions (3).

$$N_2 + NO = \text{gas observed}; \quad 2N_2 + NO = NO_2^- \text{ disappeared}$$

Therefore,

$$N_2 = NO_2^- - (\text{gas observed}), \text{ and } NO = 2 \times (\text{gas observed}) - (NO_2^- \text{ disappeared})$$

Effect of Pyridine Nucleotides on Nitrite Reductase ($NO_2^- \rightarrow NO$)—The best results were observed when the fraction obtained at 0.55 ammonium sulfate saturation was reprecipitated two or three times at 0.7 saturation and dialyzed overnight. Fig. 1 shows that with Difco yeast extract as the hydrogen donor substrate the addition of 0.46 μmole of DPN[1] resulted in considerable increase in enzyme activity over the control lacking this cofactor. The extent of stimulation indicated that DPN was functioning in a catalytic capacity and that the extract contained the necessary dehydrogenase system capable of generating DPNH. The use of alkaline sulfite as an NO-trapping agent showed that the nucleotide stimulated the formation of a considerable amount of nitric oxide. Little or no nitric oxide was trapped in the absence of the nucleotide. When succinate was used as the donor substrate, similar stimulation was obtained with a catalytic amount of DPN.

It was further apparent that the fractions contained glucose-6-phosphate dehydrogenase as well as the other dehydrogenases. The fraction collected between 0.4 and 0.7 saturation utilizes TPN more effectively than DPN when glucose-6-phosphate (20 μmoles) is added.

By repeating the ammonium sulfate precipitation three to five times and dialysis of the enzyme preparation, a point could sometimes be reached at which the enzyme showed no activity without added pyridine nucleotide, even after prolonged incubation (Tables I and III). Upon the addition of DPN or TPN, a stimulation of NO and N_2 production occurred. This was estimated by absorption into alkaline sulfite or calculated from the amount of nitrite which disappeared and the total volume of gas formed. Representative experiments on different enzyme preparations with TPN as cofactor are presented in Table I. In each instance there was a net increase in nitrite disappearance and in NO and N_2 production over the control lacking TPN.

Effect of Flavin Nucleotides on Nitrite Reductase—When a fraction ob-

tained between 0.4 and 0.7 ammonium sulfate saturation was used, the addition of catalytic amounts (0.1 μmole) of FMN or FAD to the reaction mixture containing TPN resulted in stimulation of nitrite disappearance to the extent of 25 per cent over that observed when TPN was the only cofactor present. Similarly NO formation from nitrite was stimulated to about the same extent. This could only be observed when NO was
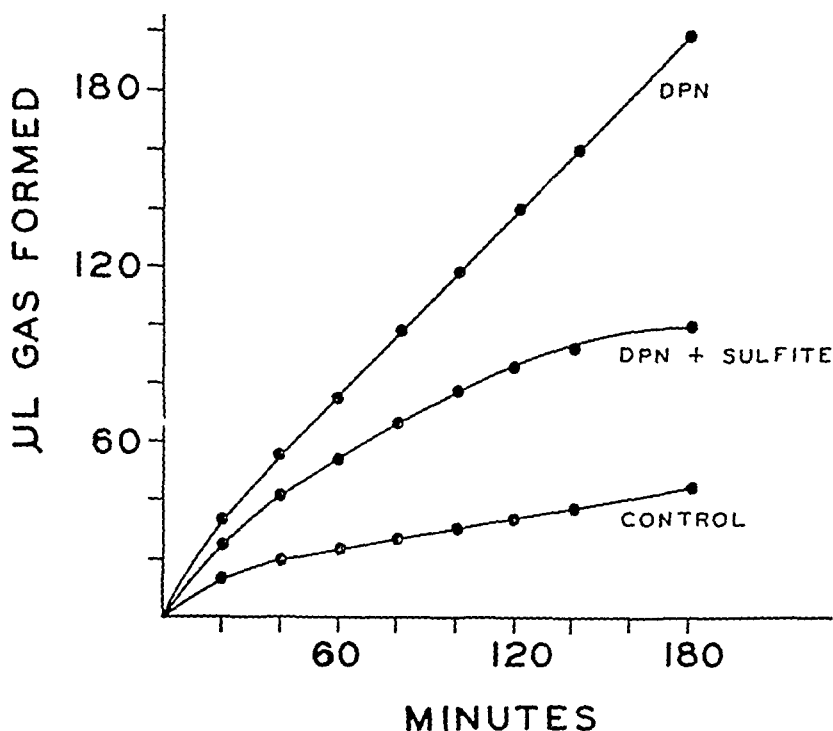
Fig. 1. Effect of DPN on gas formation from nitrite. The reaction mixture consisted of enzyme (0.55 fraction) 29.6 mg., phosphate buffer, pH 6.8, final concentration 0.08 M, in the main compartment. Difco yeast extract 4 mg., DPN 0.46 μmole, and sodium nitrite 20 μmoles were tipped in from one side arm at zero time. Total volume of the reaction mixture 2.6 ml. The other side arm contained 0.2 ml. of either water or 5 per cent of sodium sulfite in 0.1 N NaOH. 0.2 ml. of 20 per cent KOH in the center well; bath temperature 30°; gas phase pure nitrogen.

trapped with alkaline sulfite. In the absence of the trapping agent, NO was rapidly converted to N_2, owing to the stimulation of nitric oxide reductase present in the extract by the flavin nucleotides (7).

The effect of the flavin nucleotides in stimulating nitrite reductase became much more pronounced when the enzyme was further resolved with acid treatment. Table II shows that nitrite utilization was *increased* about 2-fold. The amount of NO trapped in sulfite was correspondingly increased. Formation of nitrogen gas was also stimulated because of the stimulation of nitric oxide reductase, which converted soluble NO to N_2.

Effect of Metal Ions on Nitrite Reductase—A number of metal-binding agents were tested for possible inhibition of denitrification. Only salicyl-aldoxime, KCN, potassium ethyl xanthate, and sodium diethyldithio-carbamate showed effective inhibition. All inhibition studies were performed by preincubation of the enzyme preparation with the inhibitors for 1 hour before substrate addition.

TABLE I

Effect of TPN on Formation of Nitrogen and Nitric Oxide from Nitrite

Experiment No.	TPN	Gas formed	NO₂⁻ disappearance	NO calculated*	N₂ calculated*
		μl.	μmoles	μmoles	microatoms
1	—	32.9	1.82	1.12	0.70
	+	104	5.2	4.10	1.10
2	—	28.1	1.35	1.16	0.19
	+	98.2	4.72	4.05	0.67
3	—	0	0	0	0
	+	110.7	5.42	4.46	0.96
4	—	23.3	1.03	1.03	0
	+	93.3	4.75	3.58	1.17
5	—	60.7	3.78	1.63	2.15
	+	116.6	6.08	4.33	2.75

The main compartment contained phosphate buffer, pH 6.8, final concentration 0.08 M, MgCl₂ 10 μmoles, isocitric dehydrogenase 1.0 mg., enzyme, glucose-6-phosphate 20 μmoles, isocitrate 10 μmoles, and sodium nitrite 10 μmoles in one side arm; the other contained TPN 0.1 μmole, total volume 3.0 ml. All substrates were tipped in at zero time. 0.2 ml. of 20 per cent KOH in the center well; gas phase pure nitrogen; bath temperature 30°; incubation period 3 hours. Enzyme (0.4 to 0.7 fraction) precipitated three to five times at 0.8 of ammonium sulfate saturation and dialyzed against cold distilled water for 24 to 90 hours. The amount of enzyme added in Experiments 1, 3, and 4 was 24 mg.; in Experiment 2, 20 mg.; in Experiment 5, 31 mg. Isocitric dehydrogenase was similarly precipitated and dialyzed.

* See the text.

At concentrations of 5 to 7 × 10⁻² M, cyanide produced over 90 per cent inhibition of nitrite disappearance or gas formation. At 1 × 10⁻² M about 40 per cent inhibition of both activities occurred, and at 1 × 10⁻³ M or below very little inhibition was observed. Sodium diethyldithiocarbamate and potassium ethyl xanthate gave relatively high inhibition at 1 × 10⁻² M. It appeared that the nitric oxide reductase was inhibited more strongly by the carbamate than was the nitrite reductase.

At concentrations of 2 × 10⁻³ M, pyrophosphate, o-phenanthroline, and Versene-Fe-3-specific caused little or no inhibition. At 10⁻³ M EDTA and 8-hydroxyquinoline also produced no inhibition. With higher concentra-

tions of EDTA, pyrophosphate, and thiourea there was some inhibition. Dialysis of the enzyme preparation against 10^{-2} M solutions of cyanide and pyrophosphate resulted in an almost complete inhibition of the enzymes, and the addition of various metals was not successful in restoring the ac-

TABLE II

Effect of FMN or FAD on Nitrite Disappearance by Nitrite Reductase

Cofactors added			NO_2^- disappearance	NO absorbed into sulfite	N_2 formed
			μmoles	*μmoles*	*microatoms*
0.05 μmole TPN			5.87	3.97	1.90
0.05 "	"	+ 0.1 μmole FMN	12.34	7.44	4.90
0.05 "	"	+ 0.1 " FAD	11.00	6.77	4.20
0.1 "	"		6.78	5.44	1.34
0.1 "	"	+ 0.1 μmole FMN	12.93	8.10	4.83
0.1 "	"	+ 0.1 " FAD	11.81	7.61	4.20
0.2 "	"		7.43	5.47	1.96
0.2 "	"	+ 0.1 μmole FMN	13.40	8.85	4.55
0.2 "	"	+ 0.1 " FAD	12.93	7.23	5.70
0.2 "	"	+ 0.3 " FMN	13.46	5.04	8.42
0.2 "	"	+ 0.2 " FAD	13.36	7.40	5.95

The reaction mixture consisted of 25.4 mg. of enzyme, 10 μmoles of $MgCl_2$, 10 μmoles of $MnCl_2$, tris(hydroxymethyl)aminomethane buffer, pH 7.0, final concentration 0.08 M, in the main compartment; 0.05 to 0.2 μmole of TPN, 0.1 to 0.2 μmole of FMN or FAD, and 20 μmoles of $NaNO_2$ in one side arm. The other side arm contained 0.5 ml. of 5 per cent Na_2SO_3 in 0.1 N NaOH, and the center well 0.3 ml. of 10 per cent Na_2SO_3 in 0.1 N NaOH with filter paper strips. Substrates and cofactors in the side arm were tipped in at zero time. Total volume 3.0 ml.; bath temperature 30°; gas phase pure nitrogen; incubation period 180 minutes. The enzyme used in this experiment was prepared as follows: To the enzyme preparation HCl was added to 0.0015 M; then the solution was quickly brought to 0.5 saturation with solid ammonium sulfate in the cold and left for 18 hours at −3°. It was then precipitated at 0.8 saturation and dialyzed for 48 hours at 2–4° with several changes of water. Control experiments showed that the sulfite solution used as above was sufficient to absorb the CO_2 produced.

tivity. Dialysis of the enzyme preparations against 0.01 M NH_4OH for several days showed no inhibitory effect, and no stimulation was obtained by addition of metals.

The addition of Cu^{++}, Fe^{+++}, or Fe^{++} reversed the inhibition produced by salicylaldoxime and sodium diethylthiocarbamate not only in terms of nitrite disappearance, but also in the conversion of nitric oxide to nitrogen. The latter effect was more distinct than the former. The reversal effect

was best with copper and least with ferrous ion. Other metals tested, Mn^{++}, Co^{++}, Zn^{++}, Ca^{++}, MoO_3, and $MoO_4^=$, had no effect.

The inhibition of denitrification by metal-binding agents and the counteracting effect of copper and iron ions strongly suggested the possible participation of these metals in the denitrifying processes. The experiments that follow show definitely that copper and iron are obligatory activators of this system, possibly in the rôle of electron transport.

TABLE III

Effect of Cu^{++}, Fe^{+++}, and Fe^{++} on Nitrite Reductase (Salicylaldoxime-Treated)

Metal	NO_2^- disappearance	NO absorbed into sulfite
	$\mu moles$	$\mu moles$
None. ..	8.10	3.39
5×10^{-4} M Cu^{++} .	10.26	7.64
5×10^{-4} " Fe^{+++}.	9.75	6.59
5×10^{-4} " Fe^{++}.	9.10	5.56
5×10^{-4} " Co^{++}.	5.90	2.74
5×10^{-4} " Zn^{++}..	6.20	1.87
2.5×10^{-4} M MoO_3	8.00	3.09
5×10^{-4} M MoO_4^-	8.50	3.68
5×10^{-4} " Ca^{++}	8.60	3.04

The enzyme was dialyzed against 1×10^{-2} M salicylaldoxime for 72 hours and then against frequent changes of demineralized water at $2-4°$ for 100 hours. The reaction mixture consisted of 21.2 mg. of enzyme, 0.1 $\mu mole$ of TPN, 0.1 $\mu mole$ of FMN, 5 $\mu moles$ of $MnCl_2$, tris(hydroxymethyl)aminomethane buffer at pH 7.0, final concentration 0.08 M, in the main compartment; 40 $\mu moles$ of l-malate, 20 $\mu moles$ of $NaNO_2$; metals as indicated in one side arm. The other side arm contained 0.5 ml. of 5 per cent Na_2SO_3 in 0.1 N NaOH, and the center well 0.3 ml. of 10 per cent Na_2SO_3 in 0.1 N NaOH with filter paper strips. Substrates and cofactors in the side arm were tipped in at zero time. Total volume 3.0 ml.; temperature 30°; gas phase nitrogen; incubation time 180 minutes. Control experiments showed that the sulfite solution used as above was sufficient to absorb the CO_2 produced.

Fractionated extracts were dialyzed for several days against 1×10^{-2} M salicylaldoxime or diethyldithiocarbamate in order to remove bound metals. This was followed by exhaustive dialysis against cold demineralized water for several days. The enzyme preparations thus obtained were used for studies of metal activation.

The addition of Cu^{++}, Fe^{+++}, or Fe^{++} ions to those preparations treated with salicylaldoxime resulted in an increase in NO production and nitrite disappearance when the former was trapped in sulfite. Table III contains representative data showing such an effect. Cobalt, molybdenum, and calcium have no effect, and zinc is definitely inhibitory. The highest activation obtained with copper ions was 40 to 50 per cent at concentra-

tions of 5×10^{-4} M Cu^{++}. The stimulatory effect of copper is very likely greater in magnitude than is apparent in these studies, since the inhibitory concentration is not far removed from that producing stimulation.

DISCUSSION

Because of the presence of very active DPNH and TPNH oxidases in the extracts it was not possible to perform these studies spectrophotometrically. These oxidases were not inhibited by high concentrations of cyanide (0.05 M). It was not possible to determine whether both TPN and DPN functioned as the electron carriers in the reduction of nitrite to nitric oxide or whether the presence of a transhydrogenase was responsible for the effectiveness of both (8). In contrast to the reduction of nitrite to hydroxylamine by enzymes in the *Neurospora*, soy bean leaves, and *Bacillus pumilus* (9–11), the enzymes from *P. stutzeri* produce nitric oxide as the first recognizable product of nitrite reductase. The flavin nucleotides are most likely obligatory carriers in the electron transport from reduced pyridine nucleotides to nitrite. It has not been possible, however, to show an absolute dependence on the flavin, as was possible with the pyridine nucleotides. The results on the metal ion effect show undoubtedly a stimulation of nitrite reductase activity. Whether that is due to a rôle of the metal in the electron transport or to another metal function (3) remains to be determined. This enzyme, like nitric oxide reductase (7), may, nevertheless, be classified as a metalloflavoenzyme.

SUMMARY

A method for obtaining cell-free extracts of *Pseudomonas stutzeri* capable of producing NO and N_2 from nitrite has been presented. Ammonium sulfate fractions showed marked stimulation of activity when DPN or TPN was added in catalytic amounts with the necessary hydrogen donor substrates. FAD and FMN also stimulated nitrite reductase. Of the metal ions tested only Cu^{++}, Fe^{++}, and Fe^{+++} produced definite stimulation.

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COFACTOR REQUIREMENTS FOR ENZYMATIC
DENITRIFICATION

II. NITRIC OXIDE REDUCTASE*

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The reduction of nitrite to nitric oxide has been shown to require DPN
or TPN, FAD or FMN,[1] copper, and iron as electron carriers. This was
determined by the stimulation of nitrite disappearance as well as by in-
creased nitric oxide formation. Evidence was also presented indicating
that the reduction of NO to N_2 was stimulated by the same cofactors.
The evidence was based on the stimulation of N_2 formation, with con-
comitant acceleration of NO disappearance. The latter was estimated in-
directly from the amount of nitrite disappearance and the total volume of
gas obtained. This report presents data supporting these conclusions,
obtained by direct observation of NO reduction to N_2. The NO was gen-
erated chemically or obtained commercially. A preliminary account of
this was reported earlier (1, 2).

Materials and Methods

Nitric oxide reductase (NO → N_2) was obtained from extracts of *Pseu-
domonas stutzeri* fractionated with ammonium sulfate at 0.4 to 0.7 satura-
tion and repeatedly precipitated to effect a resolution of the enzyme. The
details of the procedure were essentially as described previously (3).

In the early phase of this study nitric oxide was generated by dropping
5 N nitric acid on copper turnings under N_2 gas (4). Most of the studies,
however, were carried out with commercially available nitric oxide (Mathe-
son). In order to prevent the formation of NO_2 from NO and oxygen, all
manometers were flushed with N_2 free of O_2 for 35 minutes prior to the in-
troduction of NO. NO_2 reacts with water to form a mixture of nitric and
nitrous acids, which lowers the pH and denatures the enzyme. Any possi-
ble NO_2 contamination of the gas must therefore be removed by previous

[1] The following abbreviations are used: diphosphopyridine and triphosphopyri-
dine nucleotides, DPN and TPN, respectively; flavin mono- and flavin dinucleotides,
FMN and FAD, respectively; tris(hydroxymethyl)aminomethane, Tris.

passage through two successive Erlenmeyer flasks containing 200 ml. of water or more. In practice a mixture of NO and N_2 (approximately 25 and 75 per cent, respectively) was used in the gas phase. In calculating manometer constants, a solubility coefficient of 0.04 (at 30°) was used for NO (5). The pH optimum of the over-all system of NO reduction to N_2 with glucose-6-phosphate and TPN as electron donor system, was found to be around pH 8.0. The buffer system used was a mixture of Tris, 0.08 M, and phosphate, 0.1 M.

Results

Effect of DPN or TPN—Enzyme preparations obtained as described earlier were reprecipitated two or more times by ammonium sulfate at 0.8 saturation and dialyzed against frequent changes of cold distilled water for 12 to 18 hours. Such preparations showed little or no uptake of NO, even when malate, glucose-6-phosphate, or glutamate was added. However, the addition of TPN or DPN with these hydrogen donor substrates resulted in a marked increase in nitric oxide uptake (Table I). When no specific dehydrogenases were added, TPN was usually more active than DPN, indicating probably that the TPN-linked dehydrogenases were more active or abundant in the enzyme preparation. Occasionally when malate was the hydrogen donor substrate, DPN showed as much activity as TPN.

When the enzyme preparation was fortified with DPN and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (6), a far greater stimulation resulted with DPN than with TPN (Table I).

Effect of Flavin Nucleotides—FAD and FMN were shown earlier (3) to increase N_2 formation from nitrite and, concomitantly, resulted in a decrease of NO accumulation in the gas phase. The result of adding either of these to a partially resolved enzyme is reported in Table II. Unfractionated extracts show no stimulation by either nucleotide, whereas the fractionated enzyme shows a definite requirement for full activity, suggesting that ammonium sulfate fractionation, repeated precipitation, and prolonged dialysis are effective in producing at least a partial resolution of the enzyme.

Effect of Metals on Nitric Oxide Reductase—In studies of the over-all enzymatic reduction of nitrite to nitrogen, it was found that the reduction of nitric oxide to nitrogen was the most sensitive step to the action of metal-binding agents such as cyanide, salicylaldoxime, and sodium diethyldithiocarbamate. The effect of these agents was again studied with NO as the substrate. Demineralized water, as described in Paper I, was used in all reagents. Preincubation of the enzyme with the metal binders for a period of 1 hour was performed before any activity measurements were started (Table III). At 1×10^{-2} M and 5×10^{-3} M, sodium diethyldithiocarba-

mate showed an inhibition of nitric oxide uptake of about 50 and 40 per cent, respectively. The carbamate was a good inhibitor only when freshly prepared, since the reagent was not stable on storage. On the other hand,

TABLE I

Effect of Pyridine Nucleotides on Nitric Oxide Reductase

Additions to reaction mixture	NO converted to N_2 in 30 min.
	μmoles
None ...	0.00
Glucose-6-phosphate...	0.27
" + TPN	10.94
" + DPN	19.54
l-Malate + TPN ...	3.61

The reaction mixture consisted of enzyme 26.8 mg., glucose-6-phosphate dehydrogenase from *L. mesenteroides* 5 mg., Tris buffer of pH 7.9, final molarity 0.08, in the main compartment; TPN and FMN 0.1 μmole each and $MgCl_2$ 10 μmoles in one side arm, and 30 μmoles of glucose-6-phosphate or *l*-malate in the other. Total volume of the reaction mixture 3.0 ml.; 0.2 ml. of 20 per cent KOH in the center well; bath temperature 30°; gas phase approximately 25 per cent NO in pure nitrogen. The reaction was started by tipping in the contents of the side arms.

TABLE II

Effect of Flavin Nucleotides on Nitric Oxide Reductase

Additions to reaction mixture	NO converted to N_2 in 180 min.	
	Experiment 1	Experiment 2
	μmoles	*μmoles*
None . .	0.6	0.2
TPN .	7.8	10.0
" + FMN .	12.2	16.4
" + FAD		15 0
DPN	0 2	
" + FMN ..	1 8	

The reaction mixture consisted of *d*-isocitric dehydrogenase 1 mg., enzyme 25 5 mg , Tris buffer of pH 7.9, final molarity 0.08, in the main compartment; TPN or DPN 0.1 μmole, FMN or FAD 0.1 μmole, and $MgCl_2$ 10 μmoles in one side arm; glucose-6-phosphate 20 μmoles and *d*-isocitrate 10 μmoles in the other side arm. The other conditions were the same as in Table I.

salicylaldoxime, which is stable for a long period in concentrations of 1×10^{-2} M and 5×10^{-3} M, gave about 45 and 35 per cent inhibition, respectively.

When the enzyme was dialyzed at 0° against either of these metal-bind-

ing agents and subsequently against demineralized water, there was very definite stimulation of enzyme activity by the addition of copper or iron.

TABLE III

Effect of Metal-Binding Agents on Nitric Oxide Reductase

Inhibitor	Concentration	NO converted to N_2 in 180 min.		
		Experiment 1	Experiment 2	Experiment 3
	M	$\mu moles$	$\mu moles$	$\mu moles$
None..............................		6.70	4.90	3.77
Diethyldithiocarbamate..................	1×10^{-2}	3.75	1.86	
"	5×10^{-3}	4.82	3.18	
Salicylaldoxime........................	1×10^{-2}	3.50		2.38
"	5×10^{-3}	3.65		2.95

The reaction mixture consisted of the enzyme, 1 mg. of *d*-isocitric dehydrogenase, inhibitor as indicated, phosphate buffer of pH 7.0, final molarity 0.08, in the main compartment; TPN and FMN 0.1 μmole each and 10 μmoles of $MgCl_2$ in one side arm; 20 μmoles of glucose-6-phosphate and 10 μmoles of *d*-isocitrate in the other side arm. Other conditions as in Table I. The amount of enzyme was 22.6 mg. in Experiment 1 and 16.8 mg. in Experiments 2 and 3.

TABLE IV

Activation by Cu^{++} of Nitric Oxide Reductase Treated with Metal-Binding Agents

Concentration of Cu^{++}	NO converted to N_2 in 180 min.			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
M	$\mu moles$	$\mu moles$	$\mu moles$	$\mu moles$
None	6.8	6.6	6.4	6.2
1×10^{-3}	5.3	5.9	9.8	9.0
5×10^{-4}	8.3	8.1	9.4	8.0
1×10^{-4}	9.8	9.3	7.3	6.8

The reaction mixture consisted of buffer, 0.08 M final concentration, enzyme, *d*-isocitric dehydrogenase 1 mg., $MgCl_2$ 10 μmoles, and $CuSO_4$ as indicated, in the main compartment; TPN and FMN or FAD 0.1 μmole each in one side arm; glucose-6-phosphate 20 μmoles, *d*-isocitrate 10 μmoles, and *l*-malate in the other side arm. Other conditions as described in Table I. The enzyme was treated with 1×10^{-2} M of either metal-binding agent for 48 hours and dialyzed against demineralized water for 72 hours. In Experiments 1 and 2, Tris buffer, pH 7.9 and enzyme 17.8 mg., treated with salicylaldoxime. In Experiments 3 and 4, phosphate buffer, pH 7.0 and enzyme 30.6 mg., treated with diethyldithiocarbamate. FMN was used in Experiments 1 to 3 and FAD in Experiment 4.

With untreated enzyme preparations, the stimulation was not observable, and copper ions often showed an inhibitory effect. The prolonged dialysis used in these experiments resulted in a considerable loss of enzyme activity.

It was therefore necessary to concentrate the enzyme solution by evaporation in the cold before use.

In the experiments reported in Table IV, the treated enzyme showed a maximal activation of approximately 40 to 50 per cent at concentrations of Cu^{++} 1 to 10 $\times$ 10⁻⁴ M when all other known cofactors were added to the reaction.

When FMN was replaced by FAD in the same concentration, stimulation within the same range was obtained.

TABLE V

Effect of Various Metals on Nitrite Oxide Reductase Treated with Salicylaldoxime

Metal	Concentration	NO converted to N₂ in 180 min.
	M	μmoles
None		7.8
Cu^{++}.	1 $\times$ 10⁻⁴	10.9
Fe^{++}..	2 $\times$ 10⁻⁴	9.8
" ..	2 $\times$ 10⁻⁴	9.4
MoO_4^-	5 $\times$ 10⁻⁴	7.7
MoO_3	1 $\times$ 10⁻⁴	7.8
Mn^{++}	5 $\times$ 10⁻⁴	7.9
Zn^{++}	5 $\times$ 10⁻⁴	8.3
Co^{-+}	2 $\times$ 10⁻⁴	7.8

The reaction mixture consisted of enzyme 23.6 mg., d-isocitric dehydrogenase 1 mg., Tris buffer of pH 7.9, final molarity 0.08, and $MgCl_2$ 10 μmoles, in the main compartment; TPN and FMN 0.1 μmole each; metal as indicated in one side arm; glucose-6-phosphate 20 μmoles, l-malate 10 μmoles, and d-isocitrate 10 μmoles in the other side arm. Other conditions as described in Table I. The enzyme was treated with 1 $\times$ 10⁻² M salicylaldoxime for 48 hours and dialyzed against demineralized water for 10 days.

In Table V, the effects of other metals were compared by using an enzyme preparation treated with 1 $\times$ 10⁻² M salicylaldoxime. Cu^{++} at 5 $\times$ 10⁻⁴ M showed 58 per cent stimulation, and ferric and ferrous ions at 2 $\times$ 10⁻⁴ M gave 27 and 15 per cent activation, respectively. MoO_4^-, MoO_3, Mn^{++}, and Zn^{++} at 5 $\times$ 10⁻⁴ M and Co^{++} at 2 $\times$ 10⁻⁴ M had no effect on enzyme activity.

DISCUSSION

It appears evident from the preceding results that the reduction of nitric oxide to nitrogen requires for full activity either TPN or DPN and FMN or FAD, as well as copper and iron ions. At no time was it possible to show any additive effects between DPN and TPN or between FAD and

FMN. In general the nitric oxide reductase is more amenable to resolution by repeated ammonium sulfate precipitation than is nitrite reductase.

It has not been possible to show definitely any rôle for cytochrome in extracts. The enzyme contains substantial amounts of cytochrome which cannot be readily separated. That this compound plays a part in the electron transfer during denitrification is strongly suggested by its accumulation in large quantities during anaerobic growth of the organism in the nitrate or nitrite medium. Furthermore, it was found that intact cells show an absorption band at 556 mμ, which disappears when the cells are exposed to nitrite or nitrate (7).

The demonstration that NO is reduced to N_2 does not exclude the formation of N_2 from other intermediates in denitrification. However, until that has been shown to be the case, it is reasonable to assume that NO is on the main pathway of denitrification. The electron transfer mechanism may be visualized as follows:

$$H^+ + \begin{matrix} TPNH \\ or \\ DPNH \end{matrix} \rightarrow FAD \rightarrow Cu^{++} \text{ or (and) } Fe^{+++} \text{ (cytochrome)} \rightarrow NO$$
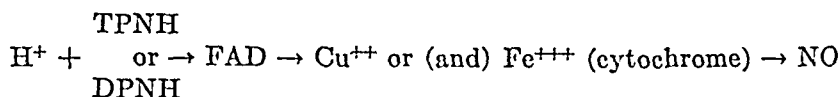
SUMMARY

The enzymatic reduction of NO to N_2 requires the presence of a pyridine and a flavin nucleotide as well as copper or iron ions for maximal activity.

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A FURTHER STUDY OF CRYSTALLINE d-UROBILIN*

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The isolation of a crystalline dextrorotatory d-urobilin hydrochloride from infected fistula bile was reported from this laboratory a number of years ago (1). Recently this substance has also been obtained from the feces of patients whose fecal flora has been altered by Aureomycin or Terramycin (2). This has been confirmed by James et al. (3). In these earlier studies the substance was isolated by means of the procedure described in 1934 (4) for isolation of stercobilin. As well as being long and tedious, this method has never permitted preparation of the crystalline-free d-urobilin, even though the hydrochloride first obtained was repeatedly recrystallized from chloroform or acetone. The customary method of conversion of the hydrochlorides of stercobilin or optically inactive urobilin (i-urobilin) to the corresponding free substances by crystallization from acetone was unsuccessful in the case of the free d-urobilin. It was believed important to crystallize the free substance in order to permit comparison of its melting point and of other characteristics with those of stercobilin and i-urobilin. A new and simpler method of isolation of these three members of the urobilin group has recently been described (5), which embodies a technique of recrystallization in which chloroform is avoided, as it promotes oxidation and is present in the crystals as $CHCl_3$ of crystallization.

Methods

The hydrochlorides of stercobilin, i-urobilin, and d-urobilin, especially of the latter, are best recrystallized from methyl alcohol-ethyl acetate (5). By far the purest d-urobilin hydrochloride has been obtained by this method. The crystals are long needle-like prisms of light orange-yellow color.

The crystalline hydrobromide was readily prepared for comparative analytical purposes. The crystalline hydrochloride was dissolved in dilute Na_2CO_3 solution, followed by acidification with hydrobromic acid (1.5 sp.

gr., diluted with 4 volumes of water). The hydrobromide was then extracted with $CHCl_3$, precipitated in petroleum ether (see below), and crystallized from methyl alcohol-ethyl acetate in the same manner as the hydrochloride.

The crystalline-free *d*-urobilin is also prepared from the hydrochloride. The crystals are dissolved in a small amount of dilute sodium carbonate solution. This is placed in a separatory funnel, barely acidified with 5 per cent tartaric acid and extracted two or three times with ethyl ether to remove any mesobiliviolin that may have formed, especially if the hydrochloride has not been freshly recrystallized. The removal of mesobiliviolin is readily followed by a change in the color of the ether, the aqueous solution assuming a more orange-yellow color as the mesobiliviolin is extracted. The *d*-urobilin is then extracted from the aqueous solution by $CHCl_3$, a number of extractions being necessary to remove most of the material. The amount of chloroform is kept at a minimum and the pooled chloroform solution is filtered through chloroform-moistened paper into a large volume of dry petroleum ether (at least 10 parts of petroleum ether to 1 of chloroform). The free *d*-urobilin that precipitates almost quantitatively is collected. It is then at once redissolved in a minimal amount of methyl alcohol, which is further concentrated by brief heating in the boiling water bath under reduced pressure. To the concentrate are added a few ml. of hot acetone, the solution being held very briefly in a boiling water bath. On cooling, the substance crystallizes in long needles. Owing no doubt to the considerably greater lability of the free *d*-urobilin and its easy conversion to mesobiliviolin and glaucobilin, the yield is small. In one run, 11 mg. were obtained from 99 mg. of hydrochloride.

The dioxane-HCl reaction was employed as described for *d*-urobilin in the first report of its isolation (1). The material to be tested was dissolved in 5 ml. of pure dioxane, and 0.1 ml. of 3.0 N HCl was added. The solution was then heated in the boiling water bath, the color change being observed every 30 seconds for 3 minutes. The Lemberg color reaction with $FeCl_3$ in HCl (6) was studied with *d*-urobilin, as contrasted with *i*-urobilin; a significant difference was noted as described later.

Amalgam reductions were carried out in small volumes of solution (7).

Melting points were determined with a Fisher-Johns block. The thermometer was calibrated against a number of compounds of known melting point between 100–300°.

Optical activity was determined in a Rudolph model No. 80 high precision polarimeter, 20 cm. cells being used. Spectroscopic absorption maxima were measured in a Zeiss grating spectrometer. Infra-red spectra were recorded in a Perkin-Elmer model No. 21 spectrometer in KBr pellets.[1] X-ray crystal powder diffraction patterns were taken with a Gen-

[1] Department of Physical Chemistry, University of Minnesota. The authors

eral Electric XRD-3 unit, with the use of CuK α-radiation and a North American Phillips powder camera adapted by the Central Research Laboratories, Red Wing, Minnesota. Elementary microanalyses were carried out after the materials were dried in a high vacuum over calcium hydride at 56°.[2]

TABLE I

Melting Points (Decomposition)

	Hydrochloride	Free substance
	°C.	°C.
d-Urobilin.	162–165	172–174
i-Urobilin...........................	159–164	175–177
Stercobilin.........................	157–162	234–236

TABLE II

Elementary Analyses

		Calculated	Found
		per cent	*per cent*
$C_{33}H_{40}N_4O_6 \cdot 2H_2O$	C	63.05	63.22, 62.67, 64.97
	H	7.00	7.22, 7.07, 7.10
	N	8.91	9.30, 8.97
	H_2O	5.7	4.2*
$C_{33}H_{40}N_4O_6 \cdot HCl$	C	63.4	64.03
	H	6.57	7.03
	N	8.97	8.96
$C_{33}H_{40}N_4O_6 \cdot HBr$	C	59.2	58.09
	H	6.13	6.35
	N	8.37	8.52
	Br	11.95	10.24
	Ash		0.8

* 100° at 0.1 mm. pressure over P_2O_5. This and the corresponding C and H determinations were carried out through the courtesy of James R. Kern, General Mills Research Laboratory, Minneapolis, Minnesota.

It is noteworthy that the dioxane-HCl reaction, especially the blue component, is not as intense with the purest *d*-urobilin as with that obtained from chloroform or chloroform-acetone. The reason for this is not entirely clear, but it appears likely that certain impurities in the latter preparations catalyze oxidation to mesobiliviolin and glaucobilin. These substances are

readily identified in the reaction mixture by aluminum oxide chromatography (8). The possibility was considered that admixture with mesobiliviolin itself might be responsible for the more intense reaction observed with the crystals obtained from chloroform-acetone. However, the addi-
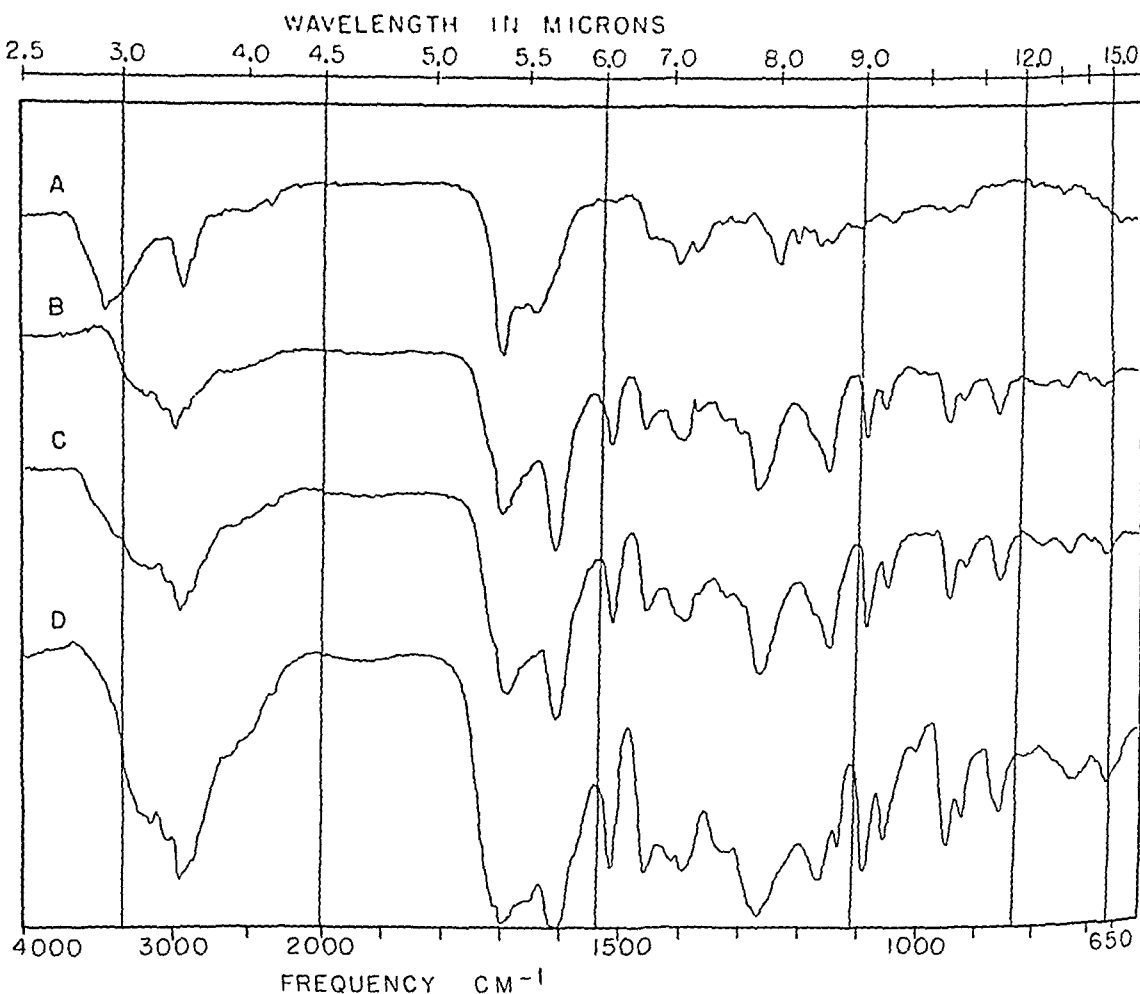
Fig. 1. Infra-red spectra of members of the urobilin group. Curve A, *d*-urobilinogen; Curve B, *d*-urobilin; Curve C, *i*-urobilin; Curve D, stercobilin.

tion of pure mesobiliviolin (8) to *d*-urobilin, carefully freed of mesobiliviolin, was ineffective in producing a more intense color reaction.

The Lemberg reaction is characterized by a deep bluish green or aqua color in contradistinction to the clear blue produced under the same conditions by *i*-urobilin. However, this is not entirely consistent. On occasion optically inactive material has yielded a green, and dextrorotatory material a blue color. Our studies to date indicate that these represent closely related oxidative stages. The melting points of *d*- and *i*-urobilin and stercobilin are given in Table I.

As with d-urobilinogen, d-urobilin is readily converted to mesobilirubinogen ($C_{33}H_{44}N_4O_6$) in the usual way (7). d-Urobilinogen is obtained, however, if the reduction is allowed to proceed for but a brief period, such as

TABLE III

Visible Absorption Spectra (Maximal) (in Dioxane)

	$m\mu$*
d-Urobilin HCl.................................	495.2 ± 0.7
i-Urobilin "	495.2 ± 0.9
Stercobilin "	492.7 ± 0.7

* Average of six readings.

TABLE IV

x-Ray Crystal Powder Patterns; Interplanar Spacings in A

Stercobilin		d-Urobilin		i-Urobilin		d-Urobilinogen	
W.	2.06	W.	1.84	W.	2.05	W.	1.78
"	2.28	"	1.91	"	2.81	"	1.87
"	2.45	"	2.02	W.M.	3.20	"	1.92
"	2.82	"	2.24	M.	3.42	W.M.	2.05
"	3.15	"	2.30	"	3.64	W.	2.13
"	3.37	"	2.41	W.	3.94	"	2.29
M.	3.50	"	2.57	M.	4.14	W.M.	2.42
M.S.	3.67	"	2.66	S.	4.71	W.	2.54
W.	3.99	W.M.	2.75	"	5.17	"	2.63
M.S.	4.22	"	2.86	W.M.	5.66	W.M.	2.74
W.M.	4.66	"	3.21	M.	7.42	M.	2.86
M.S.	4.94	M.	3.42	W.	8.32	W.	3.07
S.	5.45	"	3.62	S.	9.58	W.M.	3.39
W.	6.05	W.	3.89	M.S.	11.60	W.	3.59
M.S.	7.35	M.	4.06	M.	16.32	S.	3.74
W.M.	8.57	W.M.	4.38			M.	3.96
M.	9.80	S.	4.81			S.	4.10
"	12.96	"	5.11			"	4.26
"	16.32	W.M.	5.70			"	4.43
		W.	6.63			M.	4.76
		"	7.61			"	5.17
		"	8.65			"	5.66
		S.	9.69			M.S.	6.05
		M.	11.45			"	6.63
		"	14.69			M.	7.23
						"	7.88
						W.M.	9.58
						"	11.01
						S.	12.96

W., weak; M., moderate; S., strong.

5 to 10 minutes (see the companion paper). *d*-Urobilinogen is easily converted to *d*-urobilin, either by exposure to air or by dehydrogenation with suitable amounts of iodine (5).

The empirical formula of *d*-urobilin has not yet been determined with certainty. The elementary analyses are in best accord with the formula, $C_{33}H_{40}N_4O_6 \cdot 2H_2O$. This is in agreement with the evident water content of the compound. Because of its hygroscopic character and tendency to darken on drying, it is believed that the analyses of the free substance are not as reliable as those of the hydrochloride or hydrobromide. The analytical data are given in Table II.

This tentative formula is isomeric with mesobiliviolin on the one hand and with mesobilirubin on the other. The formula is consistent with the ease of reduction to *d*-urobilinogen (H_{42}) by amalgam and the oxidation of the latter to *d*-urobilin by I_2. The exact structure of *d*-urobilin is unknown, as is the basis for its remarkable optical activity, $[\alpha]_D^{20} +5000°$).

The infra-red spectra for *d*-urobilinogen and the three members of the urobilin group are shown in Fig. 1. It is seen that *d*- and *i*-urobilin are almost indistinguishable; the absorption of stercobilin, however, is distinctive. This has also been found to be true for the free substances. The absorption spectra of the three urobilins in the visible range are quite comparable to those observed in the infra-red (see Table III).

The three compounds are readily distinguished from one another by their x-ray crystal powder patterns (Table IV).

SUMMARY

1. A method is described for the preparation of crystalline *d*-urobilin. This involves preliminary crystallization of the hydrochloride from methyl alcohol-ethyl acetate and crystallization of the free substance from methyl alcohol-acetone.

2. A tentative empirical formula, $C_{33}H_{40}N_4O_6 \cdot 2H_2O$, is suggested for *d*-urobilin. This is believed to be derived from mesobilirubin by preliminary hydrogenation to an isomeric dihydromesobilirubin or *d*-urobilinogen. Dehydrogenation then results in the formation of the dextrorotatory urobilin.

3. The infra-red spectrum of stercobilin (*l*-urobilin) distinguishes it from *i*- or *d*-urobilin, which are themselves almost identical by this method. All three substances, however, exhibit distinctive x-ray crystal powder patterns.

4. The Lemberg iron chloride reaction characteristically exhibits a blue-green color with *d*-urobilin as contrasted with blue for *i*-urobilin, while stercobilin is unaffected.

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THE ISOLATION OF CRYSTALLINE d-UROBILINOGEN*

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Until recently there have been only two well recognized members of the urobilinogen group, mesobilirubinogen, the chromogen of i-urobilin,[1] and stercobilinogen, the leuco compound of urobilin or stercobilin. While mesobilirubinogen is readily obtained in crystalline form following amalgam reduction of bilirubin or i-urobilin, stercobilinogen has never been crystallized. Stercobilin, however, was first isolated in 1932 (1) and has been studied in considerable detail (2). As pointed out in the preceding paper (3), a third urobilin, d-urobilin, was isolated from infected fistula bile in 1942 (4). At that time the probable existence of a chromogen of d-urobilin was recognized and this was designated as d-urobilinogen, although it was not isolated. The occurrence of d-urobilin in the feces of patients treated with Aureomycin was reported in 1951 (5). Recently, while crystallizing isotopically labeled bilirubin from this source (6), it was found possible for the first time to isolate d-urobilinogen in crystalline form. The purpose of the present communication is to describe methods of isolation as well as some of the properties of this chromogen.

Methods of Isolation

d-Urobilinogen has now been crystallized from two different sources.

From Feces—d-Urobilinogen may be obtained from the feces of patients who have received Aureomycin or Terramycin. The procedure used is based on a method recently described for isolation of members of the urobilin group (7). Appropriate samples of feces (1 to 4 day collections) are repeatedly ground in a mortar with 95 per cent ethyl alcohol. After each extraction, this is filtered from the fecal residue either on a coarse sintered glass filter or with a Büchner funnel layered with infusorial earth (Hyflo).

[1] Optically inactive.

The combined alcoholic extracts are then run through a column of aluminum oxide (Baker and Adamson) previously saturated with alcohol. The urobilinogen is retained on the column and subsequently is eluted with water. The eluate is weakly acidified with glacial acetic acid and repeatedly extracted with petroleum ether. The combined petroleum ether extracts are washed several times with water, filtered through a petroleum ether-moistened filter, and concentrated to a small volume under reduced pressure. From this solution a light yellow crystalline precipitate forms.

Further purification is accomplished by chromatography. The yellow precipitate is dissolved in 2 to 3 ml. of chloroform, to which are added approximately 50 ml. of petroleum ether. If slight precipitation occurs, the solution is filtered. It is then poured on a large column of ordinary powdered (cane) sugar previously saturated with petroleum ether. The column is developed with petroleum ether. Two yellow zones appear, one at the top of the column and one nearly at the head of the eluting fluid. After full development, the colorless portion between the two yellow zones is cut out and dissolved in a small amount of water. This solution is weakly acidified with glacial acetic acid and extracted several times with petroleum ether. The combined extracts are then concentrated to a small volume under reduced pressure. On standing, colorless crystals separate. Recrystallization may be carried out from petroleum ether, chloroform-petroleum ether, or ethyl acetate. Yields of approximately 15 per cent of the original total fecal urobilinogen, as based on the Ehrlich reaction (8), are obtained.

From d-Urobilin—*d*-Urobilinogen may also be obtained by partial reduction of crystalline *d*-urobilin according to the following procedure. 100 mg. of crystalline *d*-urobilin hydrochloride (3) are dissolved in 0.2 ml. of 0.1 N NaOH, to which 0.8 ml. of water and 5 gm. of 4 per cent sodium amalgam are added. The mixture is shaken in a small container for 10 to 15 minutes. At the end of that time the sodium amalgam is removed and the solution diluted to 100 ml. with water. After acidification with glacial acetic acid, the water phase is repeatedly extracted with petroleum ether. Thereafter, the procedure is identical with that used in the isolation of *d*-urobilinogen from feces, except that chromatography is unnecessary to obtain the pure crystalline material. If reduction is continued for 30 to 60 minutes, mesobilirubinogen is consistently formed.[2] By the shorter reduction, however, crystalline material is obtained which is identical with

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[2] Ferrous hydroxide reduction, as in the regular quantitative procedure for urobilinogen in urine or feces (8), inconsistently converts *d*-urobilin or *d*-urobilinogen to mesobilirubinogen. The reduction usually occurs when crystalline material is used, but appears to be prevented or partially hindered in many instances by impurities in urine or feces. This is receiving further study.

the d-urobilinogen isolated from feces. The yield of d-urobilinogen averages 30 to 50 per cent of the weight of the original d-urobilin.

Proof that this substance is d-urobilinogen was afforded by the ease of oxidation to d-urobilin. 21.4 mg. of d-urobilinogen were dissolved in a few ml. of ethyl acetate and this was mixed with 500 ml. of petroleum ether. Dehydrogenation to urobilin was then carried out with iodine as recently described (7). A yield of 9.5 mg. of crystalline d-urobilin was obtained. The material was identified as d-urobilin by the dextrorotation ($[\alpha]_D^{20}$ +5000°) and characteristic blue reaction in the dioxane-HCl test (4).

FIG 1. Crystals of d-urobilinogen. a, from petroleum ether; b, from chloroform-petroleum ether; × 120.

Properties

d-Urobilinogen is a white powder which consists of long needles when crystallized from petroleum ether or ethyl acetate (Fig. 1, a), and of nearly square plates when crystallized from chloroform-petroleum ether (Fig. 1, b). With the latter method the yield is smaller but the crystals are larger and better formed. The compound has the following properties.

It is readily soluble in chloroform, ethyl ether, acetone, benzene, glacial acetic acid, ethyl alcohol, and dilute alkali. It is less soluble in ethyl acetate, petroleum ether, and mineral acids, and much less soluble in water.

The crystals from ethyl acetate and petroleum ether first shrink at 142–145°, but liquefaction is not complete until about 175°. The crystals from chloroform-petroleum ether melt at 110–115°. This difference, as well as the different crystal habitus, is probably due to the presence of chloroform of crystallization. When such crystals are washed with pure petroleum ether, they immediately reform as long needles and again exhibit the higher melting point.

Contrary to preliminary observations (9), d-urobilinogen has now been found to exhibit a small degree of optical activity if measured[3] in sufficient

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[3] All measurements of optical activity were made in a high precision polarimeter (O. C. Rudolph and Sons, No 21).

concentration in chloroform. Three individually prepared solutions of 1 gm. per 100 ml. gave net values of $[\alpha]_D^{20}$ +70.5°, +78.0°, and +73.5°, with an average of +74°. Corrections were made for the small amounts of *d*-urobilin which were formed after the solutions were prepared.

For comparative purposes, stercobilinogen was similarly investigated. Previous observations (10) had indicated a complete absence of optical activity, but the concentrations used never exceeded 100 mg. per cent. A petroleum ether solution of stercobilinogen (7) was concentrated to dryness under reduced pressure and the residue dissolved in chloroform to give a concentration of 2 gm. per 100 ml. With the necessary correction for the slight oxidation to stercobilin, the specific optical activity of stercobilinogen was found to be $[\alpha]_D^{20}$ −16.7°. Mesobilirubinogen, prepared in a similar manner and concentration, was optically inactive.

When heated in dioxane to which 2 drops of 3.0 N HCl have been added, *d*-urobilinogen exhibits a color play of brown-green-red-purple, the latter most frequently predominating. After standing several hours, the solution usually assumes a deep blue color similar to that which *d*-urobilin exhibits in this reaction.

The infra-red spectrum and x-ray crystal powder pattern are given in the preceding paper (3), for comparison with those of *d*-urobilin, *i*-urobilin, and *l*-urobilin or stercobilin.

No color play is exhibited with the Gmelin test.

The solution turns yellow-brown in the diazo reaction.

Separate elementary analyses[4] have been run on samples obtained from feces and from the reduction of *d*-urobilin with the following results.

Calculated ($C_{33}H_{42}N_4O_6$).	C 67.12, H 7.12, N 9.49	
" ($C_{33}H_{44}N_4O_6$).	" 66.89, " 7.43, " 9.46	
Found (from feces).	" 68.23, " 7.84, " 9.04	
	" 67.81, " 8.07, " 9.28	
Found (" *d*-urobilin).	" 68.07, " 7.80, " 8.83	
	" 67.38, " 7.37, " 9.51	

It is seen that the above analytical data are not in as close agreement as desired with the empirical formula, $C_{33}H_{42}N_4O_6$. The values for hydrogen agree better with H_{44} but the carbon analyses are believed to be most reliable, and these are in better accord with a formula having H_{42}. Furthermore, it is clear that *d*-urobilinogen is readily reduced to mesobilirubinogen, which has been synthesized (11) and has the formula $C_{33}H_{44}N_4O_6$.

Also it is easily oxidized to d-urobilin, which, as noted in the companion paper, is believed to have the formula $C_{33}H_{40}N_4O_6$. Thus the formula $C_{33}H_{42}N_4O_6$ is tentatively regarded as in best agreement with our present information.

<center>DISCUSSION</center>

Crystalline d-urobilinogen shares many of the properties of mesobilirubinogen and stercobilinogen. It is colorless, unstable, exhibits an intense Ehrlich aldehyde reaction, and is the chromogen of a urobilin.

The available evidence indicates that d-urobilinogen may be an isomer of dihydromesobilirubin ($C_{33}H_{42}N_4O_6$). It is noteworthy, however, that, while d-urobilinogen is colorless, dihydromesobilirubin is a very light yellow. The latter compound was obtained by Fischer as a side product in the catalytic hydrogenation of bilirubin (12). Several mg. of this substance have recently been prepared[5] by this process for purposes of comparison with d-urobilinogen. It also exhibits a blue color when heated in dioxane-HCl and there is no color play in the Gmelin test. Unlike d-urobilinogen, it is converted directly to mesobiliviolin when oxidized with iodine. It also differs in giving a positive diazo reaction and a violet Ehrlich aldehyde compound. Microscopically it is seen to consist of sharp, fairly short needles. It does not have a sharp melting point, but there is no change until well over 230°, in contrast to d-urobilinogen, which starts to melt at 142–145° or 110–115°, depending on the solvent from which it is crystallized.

Fischer suggested two alternative structural formulas for dihydromesobilirubin (12, 13). The possibility has been considered that one of these formulas might be that of d-urobilinogen, but this cannot be determined on the basis of the present study.

The yellow compounds which are separated from d-urobilinogen by chromatography, during its isolation from feces, have recently been identified as mesobilirubin and dihydromesobilirubin. The latter has generally been considered as a normal intermediary in the bacterial reduction of bilirubin, although purely on theoretical grounds, as it has never previously been detected in feces or intestinal content. Whether d-urobilinogen is a normal intermediary between mesobilirubin ($C_{33}H_{40}N_4O_6$) and mesobilirubinogen ($C_{33}H_{44}N_4O_6$), or an aberrant derivative formed because of an abnormal bacterial flora, is unknown. Study of this question is in progress. It is clear that d-urobilinogen is formed as the result of bacterial activity and this is of special interest since it contains less hydrogen than mesobilirubinogen, and is easily reduced to it. Since the latter is readily con-

verted by normal fecal bacteria to stercobilinogen (14, 15), it is now evident that all members of the urobilinogen group are easily formed by bacteria and that the enterogenous concept is fully supported.

SUMMARY

1. The isolation of crystalline *d*-urobilinogen is described. It has been obtained (*a*) from feces of patients treated with Aureomycin or Terramycin and (*b*) following brief amalgam reduction of *d*-urobilin. Longer reduction results in formation of mesobilirubinogen.

2. The empirical formula of the colorless *d*-urobilinogen is tentatively believed to be $C_{33}H_{42}N_4O_6$, isomeric with the light yellow dihydromesobilirubin.

3. *d*-Urobilinogen is formed by bacterial activity, but whether only an abnormal, or a transitory normal, intermediary remains to be determined.

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THE OCCURRENCE OF ERGOTHIONEINE IN PLANT MATERIAL*

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The question of the synthesis of ergothioneine by animals has been investigated in this Laboratory by the administration of isotope-labeled compounds considered to be likely precursors. No evidence for the formation of radioactive ergothioneine was obtained with C^{14}-histidine in the rat, methyl-labeled C^{14}-methionine in the rat or chicken, S^{35}-methionine in the rat, guinea pig, or pig, and S^{35}-cystine in the human (1, 2). Studies with germ-free chickens have shown that the intestinal flora does not contribute significant amounts of ergothioneine (3). We have concluded that ergothioneine is not synthesized by any species of animal so far studied and is dietary in origin.

This evidence for the non-synthesis of ergothioneine by animals has been difficult to reconcile with the fact that ergothioneine apparently occurs universally in animal bloods, since attempts by various workers over a period of years have failed to demonstrate the presence of ergothioneine in plant material (3–6). We have therefore examined oats, which have been shown to give rise to blood ergothioneine when fed to animals (6–8), and have been able to demonstrate, by isolation of the crystalline compound, that this grain does in fact contain ergothioneine.

EXPERIMENTAL

A likely explanation for previous failures to detect ergothioneine in plant materials which are known to serve as dietary sources of ergothioneine could be the occurrence of the substance in a bound form. However, we have presented evidence to indicate that the precursor effect of corn is not associated with the protein fraction of this grain (2). It seemed possible that earlier difficulties might be due to a low concentration of the substance in plants (2), coupled with the fact that many naturally occurring substances interfere with the colorimetric methods which are used for the detection of ergothioneine. Accordingly, we directed our efforts toward the detection of free ergothioneine. Oats were chosen as the starting material because Baldridge and Lewis (6) and Baldridge (7) have shown them to be particularly effective as a dietary source of blood ergothioneine, while

wheat and barley have been found to be less efficient in this respect (8), and corn has given variable results in the hands of different investigators (2, 5, 8, 9).

Rolled oats (The Quaker Oats Company, Chicago) of the kind sold for human consumption were fed to rats which had previously been depleted of blood ergothioneine by the feeding of a purified diet (10). Within 10 weeks the animals had blood ergothioneine levels of approximately 10 mg. per 100 ml. These values are considerably higher than those observed with corn (2) or other grains. However, attempts to identify ergothioneine in extracts of the oats, either directly or after alumina chromatography, were unsatisfactory because of the presence of substances which interfered with the color test with diazotized sulfanilic acid. More promising results were obtained when oat extracts were treated with basic lead acetate to remove interfering substances prior to chromatography. With this procedure we finally obtained effluent fractions which gave the characteristic magenta color of ergothioneine in the diazo test (11). This procedure was used in a quantitative fashion to follow the progress of purification during subsequent isolation experiments. The details of a fractionation procedure which led to the isolation of pure, crystalline ergothioneine are described below.

Extraction of Oats—2 kilos of rolled oats,[1] which contained 34 mg. of ergothioneine on the basis of chromatographic analysis, were ground mechanically and added to 12 liters of hot water. The mixture was heated, with stirring, on a steam bath at 80–90° for 15 minutes and then 12 liters of 95 per cent ethanol were admixed. The liquid phase was removed as completely as possible by filtration through cheese-cloth in a press and evaporated to dryness under reduced pressure. The residue weighed 65 gm. and contained 23 mg. of ergothioneine.

Treatment with Lead Acetate and Ion Exchange Resin—The 65 gm. residue was mixed with 1.5 liters of water, and 190 ml. of a basic lead acetate solution (12) were added. The precipitate was separated by centrifugation and washed once with a small volume of water. The combined solutions were adjusted to pH 2 with concentrated sulfuric acid, and the precipitated lead sulfate was separated by centrifugation. The solution was passed through a column prepared from 1.5 kilos of the acetate form of Amberlite IRA-410 exchange resin (Rohm and Haas Company, Philadelphia) and the resin was washed with 1 liter of water. The total effluent was evaporated to dryness under reduced pressure. The residue weighed 34 gm. and contained 20 mg. of ergothioneine.

[1] These oats were part of a 100 pound lot purchased from The Quaker Oats Company. Crystalline ergothioneine was also isolated in comparable yield from "old fashioned" Quaker oats purchased at local food stores.

Alumina Chromatography—The residue from the ion exchange column was dissolved in 100 ml. of water and then 300 ml. of absolute ethanol were added. The precipitate which formed was separated by centrifugation and washed twice by dissolving it in water and reprecipitating with ethanol. The combined supernatant solutions were passed through a column prepared from 4 kilos of alumina (Alcoa, grade F-20) in a column 6 cm. in diameter. The column solvent was prepared by mixing 3 volumes of ethanol with 1 volume of water. 10 liters of solvent were used, and the effluent was collected in 1 liter fractions. The major portion of the ergothioneine appeared in Fractions 7, 8, and 9. These were combined and evaporated under reduced pressure. The dry residue weighed 857 mg. and contained 20 mg. of ergothioneine.

The 857 mg. fraction was chromatographed again on alumina. In this step, 80 gm. of alumina were used and 20 ml. fractions were collected. Fractions 6, 7, and 8 contained 283 mg. of solids and 15.4 mg. of ergothioneine. These fractions were combined, evaporated to dryness, and used in the next step.

Precipitation with Cuprous Oxide—The 283 mg. of solids were dissolved in 3 ml. of 0.5 N sulfuric acid and treated with cuprous oxide according to the procedure of Hunter, Molnar, and Wight (13). The resulting precipitate was decomposed with hydrogen sulfide, the solution was adjusted to pH 7 with barium hydroxide, and the hydrogen sulfide treatment was repeated. The solution which was obtained was evaporated to dryness. The residue weighed 81.6 mg. and contained 9.2 mg. of ergothioneine.

Alumina Chromatography—The residue was chromatographed on 20 gm. of alumina with 80 per cent ethanol as the solvent. 5 ml. fractions were collected. Fractions 8 through 13 contained most of the ergothioneine. They were combined and evaporated to dryness. The residue weighed 8.6 mg. and contained 7.2 mg. of ergothioneine.

Crystallization—The dried residue was dissolved in 0.03 ml. of water, 0.25 ml. of ethanol was added, and crystallization was induced by rubbing the vessel walls with a fine glass rod. The mixture was kept at 5° for 24 hours, the mother liquor was removed, and the crystals were washed with ethanol. 6.3 mg. of needles were obtained. As a final purification step, the material was dissolved in water and passed through a small column prepared from 1 gm. of IRA-410 acetate. The solid material from the column effluent was recrystallized from 0.02 ml. of water and 0.25 ml. of ethanol. Needles weighing 5.6 mg. were obtained.

The material was indistinguishable from ergothioneine in crystalline form and solubility. Like ergothioneine, it decomposed above 250° on the micro melting point stage. The color intensity in the diazo test was the same as that given by ergothioneine. The substance showed the same

behavior as ergothioneine in ascending paper chromatography with *n*-butanol-acetic acid (R_F 0.23) and with *n*-butanol-methanol-benzene (R_F 0.45). The ultraviolet absorption spectrum in 95 per cent ethanol was similar to that described for ergothioneine (2). Infra-red spectra of the isolated material and of pure ergothioneine were obtained by incorporating finely ground 0.5 mg. samples into potassium bromide disks under high pressure. The spectra were identical (Fig. 1).
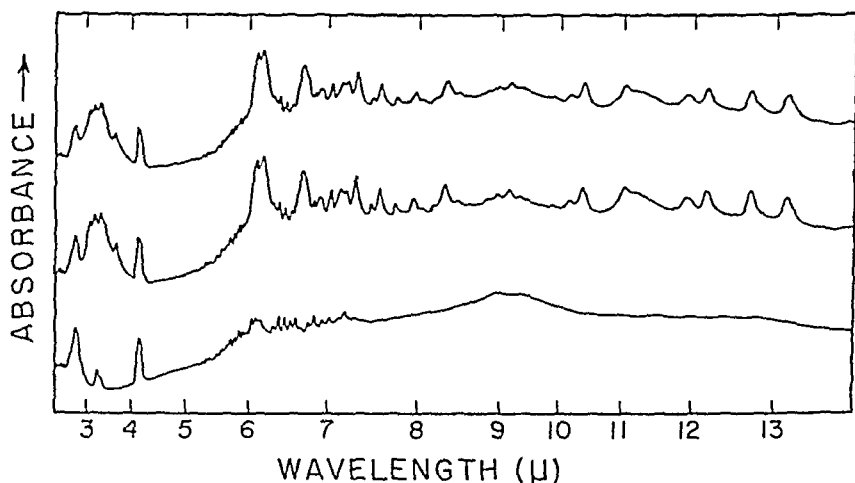
FIG. 1. Infra-red absorption spectra of KBr blank (bottom curve), ergothioneine (top curve), and the crystalline substance isolated from oats (middle curve).

DISCUSSION

The discovery of ergothioneine in a plant material clarifies considerably the question of the origin of animal ergothioneine. It makes understandable the occurrence of ergothioneine in herbivorous animals and further strengthens our belief that ergothioneine is not synthesized by animals in general. These considerations are based on the assumption that oats are not unique among edible plants in containing ergothioneine. This latter point has not been thoroughly investigated; however, other cereal grains have been shown to act as dietary precursors of blood ergothioneine, and it seems reasonable to believe that they too contain the compound. In this respect, it is of interest that the ability of corn to serve as a precursor for blood ergothioneine is also shown by aqueous acetone extracts of corn (2).

The oats used in these studies contained appreciably larger amounts of ergothioneine than the corn which was used in our earlier work (2). The value of 1.7 mg. per 100 gm. found by chemical analysis of the oats must be taken as a minimal figure for the ergothioneine content. Other samples of rolled oats of the same brand have given comparable values. It does appear, however, from the results of Potter and Franke (8) that the ergo-

thioneine content of oats may vary from crop to crop. In view of the fact that we have found that some common microorganisms synthesize ergothioneine (14) and that included in this group are fungi which are commonly present in oats, it seems quite likely that at least part of the ergothioneine of this grain is of fungal origin. This might help to explain the variations in ergothioneine content of grain from different crops.

Appreciation is expressed to Dr. Julian R. Rachele for aid in carrying out the infra-red analyses.

SUMMARY

The heretofore unexplained ability of cereal grains to serve as dietary sources of blood ergothioneine has been investigated by an examination of oats for the presence of the compound. A chemical fractionation procedure has been devised which resulted in the isolation from oats of a crystalline compound identical in all respects with ergothioneine. This discovery of ergothioneine in a plant material makes understandable its wide-spread occurrence in animal species.

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THE RELATIONSHIP BETWEEN SULFHYDRYL GROUPS AND THE ACTIVATION OF MYOSIN ADENOSINETRIPHOSPHATASE*

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Greenstein and Edsall (1) in early observations on the quantitative estimation of sulfhydryl groups in the muscle protein myosin classified the SH groups of the native protein into "free" and unavailable groups on the basis of porphyrindine titration with nitroprusside as an external indicator. In subsequent experiments, Singer and Barron (2) observed that combination of the "free" groups of myosin with p-chloromercuribenzoate (PCMB) resulted in little alteration of the adenosinetriphosphatase (ATPase) activity of myosin, while addition of PCMB equal to the total SH led to complete inhibition. It has been observed by one of us (3) that low concentrations of PCMB resulted in some increase in ATPase activity and this was also observed occasionally by Polis and Meyerhof (4).

On reexamination of the influence of PCMB titration of myosin SH, we observed that a marked increase in ATPase activity occurred when approximately one-half the sulfhydryl groups had been titrated, when Ca^{++} was employed as ATPase activator. On the other hand, only inhibition occurred when ethylenediaminetetraacetic acid (EDTA) was employed as activator (*cf.* (5, 6) for EDTA activation). The reaction of myosin with N-ethylmaleimide (NEM) gave similar results.

After the work presented here was completed, two reports (7, 8) appeared on the influence of phenylmercuric acetate and dinitrophenol on myosin ATPase. In these the authors appear to be dealing with similar phenomena.

EXPERIMENTAL

Materials and Methods

Myosin—Myosin was prepared by either of two procedures. One is a slight modification of that of Tsao (9), the other a modification of the procedure of Weber and Portzehl (10). The products obtained were indistinguishable in so far as the work presented here is concerned.

The only modification in the procedure of Tsao was the inclusion of 0.01 M EDTA in the saturated ammonium sulfate. No attempt was made to ascertain that this was essential. However, Tsao observed that his preparations were rather low in ATPase activity, whereas our preparations were of consistently high activity. In the modified Weber and Portzehl procedure, the muscle was extracted with 3 volumes of 0.5 M KCl, 0.1 M K_2HPO_4 for 20 or 30 minutes, and then treated in the Waring blendor for $\frac{1}{2}$ minute. The material was then centrifuged, and the myosins A and B were precipitated from the supernatant solution by dilution with 10 volumes of water. The redissolved precipitate (0.5 M KCl) was then adjusted to 0.28 M KCl at pH 6.7 to 6.8 and the precipitate (myosin B) removed. The myosin A of the supernatant solution was obtained by further dilution to 0.04 M KCl. The latter fraction, after resolution in 0.5 M KCl, was then subjected to $(NH_4)_2SO_4$ fractionation as in the first procedure.[1] This consisted of addition of a saturated solution of $(NH_4)_2SO_4$ (adjusted to pH 6.5 to 7.0 and containing 0.01 M EDTA) to the myosin solution to 40 per cent saturation. The precipitate formed was removed and discarded. The supernatant solution was then brought to 50 per cent saturation by further addition of the $(NH_4)_2SO_4$ solution. The precipitate was collected by centrifugation, redissolved in 0.5 M KCl, and dialyzed against 0.5 M KCl overnight. Protein was estimated from micro-Kjeldahl nitrogen determinations with correction for NH_4^+ remaining after dialysis.

Adenosine triphosphate was obtained commercially as the crystalline sodium salt.

Ethylenediaminetetraacetic acid (EDTA) was obtained commercially and used as the potassium salt (pH 7.0).

The method of Whitmore and Woodward (11) was used to prepare PCMB or to purify the material obtained commercially.

NEM was obtained commercially and purified by sublimation.

ATPase Activity—Enzymatic activity was measured in a system containing 0.001 M ATP, 0.02 M histidine (pH 7.6), either 0.005 M $CaCl_2$ or 0.001 M EDTA, and either 0.05 M KCl (when $CaCl_2$ is present) or 0.4 M KCl (when EDTA is present).

Treatment of Myosin with Sulfhydryl Reagents. PCMB—PCMB was added to myosin dissolved in 0.5 M KCl in quantities indicated in Fig. 1 and Table I. The tubes containing the treated protein were kept chilled and assayed immediately by adding aliquots containing 20 to 30 γ of protein to the medium given above (final volume 1.0 ml.).

The spectrophotometric procedure of Boyer (12) was employed to follow the reaction of protein SH with PCMB.

[1] Both myosin B and myosin A without the $(NH_4)_2SO_4$ fractionation exhibit a behavior identical to that described here.

NEM—N-Ethylmaleimide in the quantities indicated in Fig. 2 was added to myosin dissolved in 0.5 M KCl and adjusted to pH 7.2, 7.6, or 8.0 with 0.02 M histidine. The tubes containing the treated protein were allowed to stand at 0° for 2 and 20 hours before assaying aliquots containing 20 to 30 γ of protein for ATPase activity in the medium given above with volume of 1.0 ml.
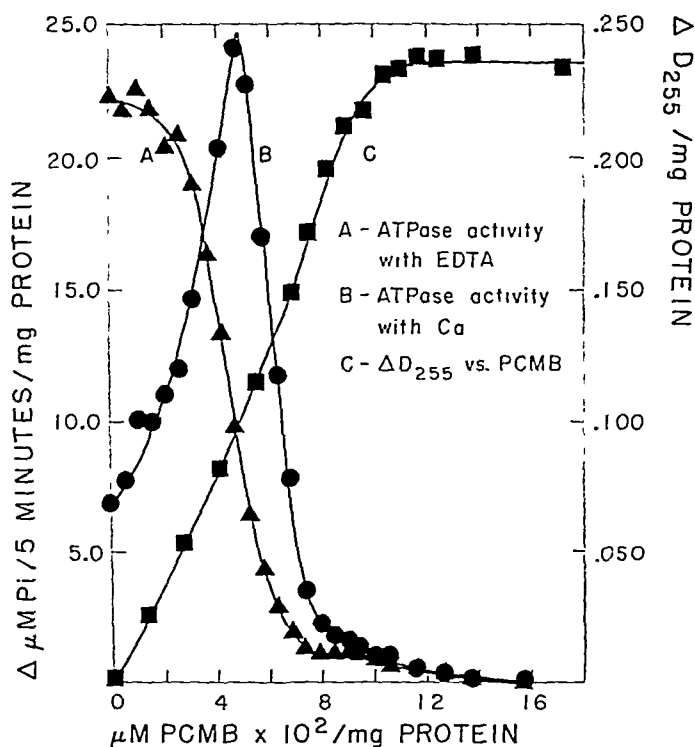
FIG. 1. Titration of myosin with PCMB. Curve A, ATPase activity in the presence of EDTA; Curve B, ATPase activity in the presence of Ca++; and Curve C, spectrophotometric titration of myosin with PCMB. For other conditions see the text.

Results

The influence of PCMB on myosin ATPase is presented in Fig. 1. Curve C represents the spectrophotometric titration of myosin SH by PCMB. The total SH is calculated to be 0.11 μmole of SH per mg. of protein. Curve B presents the behavior of ATPase activity with Ca++ as activator. Titration of about one-half the groups led to about a 4-fold increase in activity in this experiment (between 3- and 4-fold generally) with inhibition at higher concentrations of PCMB, complete inhibition corresponding to complete titration of the SH. With EDTA as activator (Curve A) only

inhibition was observed, the process being complete when only about three-fourths of the groups was titrated and the form of the curve suggests that only one-half of the groups was involved in this inactivation, and that about one-quarter of the groups is not essential for enzymatic activity. The inhibition by PCMB was reversed to some extent by other sulfhydryl compounds, as was indicated by Singer and Barron (2).

When assayed in the presence of Ca^{++} it can be seen (Table I) that the effect of an amount of PCMB (0.04 μmole per mg. of protein) approaching one-half the total SH was readily reversed by β-mercaptoethanol. However, for a higher concentration of PCMB, approaching complete inhibition, no reversal was observed. In contrast, the activity measured in the

TABLE I

Reversal of Effect of PCMB on Myosin ATPase by β-Mercaptoethanol

Activator	μmoles β-mercapto-ethanol per μmole PCMB	μmole inorganic phosphate per 5 min.		
		No PCMB	0.04 μmole PCMB per mg. protein	0.07 μmole PCMB per mg. protein
Ca^{++}	0	0.13	0.38	0.09
"	10	0.13	0.12	0.06
"	100	0.14	0.11	0.06
"	1000	0.13	0.11	0.09
EDTA	0	0.45	0.18	0.01
"	10	0.44	0.40	0.15
"	100	0.49	0.38	0.15
"	1000	0·50	0.42	0.29

presence of EDTA exhibits some reversal of the effect of both low and high PCMB concentrations by β-mercaptoethanol. The pattern of these results suggests that under the conditions (time, etc.) removal of the mercurial from the protein by even the large excesses of β-mercaptoethanol is incomplete and that those mercaptide groups reacting most readily with the β-mercaptoethanol may be sulfhydryl groups reacting first with PCMB. In the case of Ca^{++} activation, these latter SH groups appear to be inhibiting groups when free.

The results of incubating myosin with NEM at pH 7.2 and 8.0 (both at 0°) are shown in Fig. 2. Tsao and Bailey (13) observed that only about 50 per cent of the sulfhydryl groups of native myosin will react with this reagent. Comparison of the results for 2 and 20 hour incubations of myosin with NEM at 0° indicates that the reaction is slow. It is also evident from Fig. 2 that the rate and extent of the reaction are influenced by pH. Though only a fraction of the SH groups react with NEM, comparison

with PCMB indicates that a fractional reaction with either reagent may not involve the same groups and that "availability" of any particular SH group depends on the binding agent. Thus for an apparent reaction of about one-half the SH with NEM (on the basis of reagent concentration no more than one-half could have reacted) the same phenomenon of in-
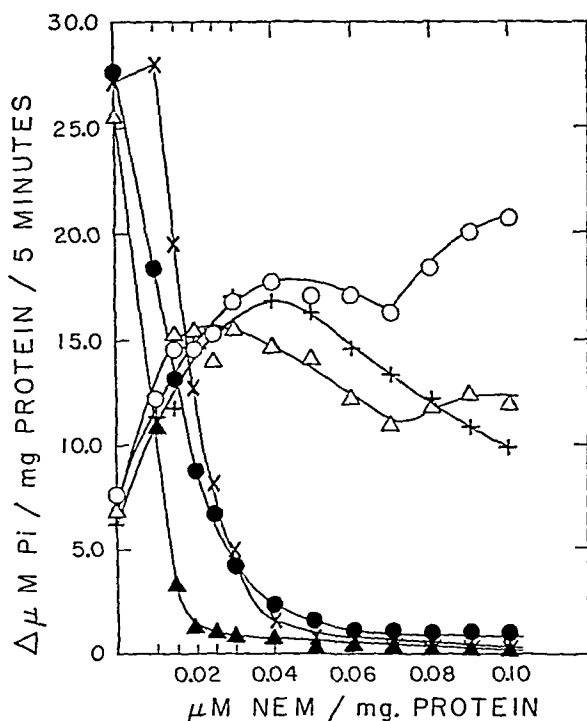
Fig. 2. Behavior of myosin ATPase treated with NEM. O, at 2 hours, pH 7.2 activity measured with Ca^{++}; ●, at 2 hours, pH 7.2 activity measured with EDTA; △, at 20 hours, pH 7.2 activity measured with Ca^{++}; ▲, at 20 hours, pH 7.2 activity measured with EDTA; +, at 2 hours, pH 8.0 activity measured with Ca^{++}; ×, at 2 hours, pH 8.0 activity measured with EDTA. For other conditions see the text.

creased enzymatic activity occurs as with PCMB in the presence of Ca. However, the magnitude of this increase and the influence of reagent concentration on its development are different from that observed with PCMB. The difference in behavior relative to the SH reagent is particularly noticeable with EDTA as activator when, with NEM, complete inhibition occurs when no more than one-quarter of the SH could have combined with the reagent, in contrast to the reaction of three-quarters of the SH groups with PCMB before complete inhibition occurs in EDTA activation.

DISCUSSION

From results with the two sulfhydryl-binding agents it appears possible to conclude that myosin possesses more than one sulfhydryl group per active center. It also appears likely that not all the SH groups are directly concerned with the enzymatic activity. Of the SH groups occurring at the active center one or more appear to participate in an interaction with some unidentified group, and this interaction prevents the enzyme from exhibiting its potential maximal activity in the presence of Ca as activator.

The behavior of the enzyme in the presence of EDTA as contrasted to that in the presence of Ca suggests that EDTA may interfere with this sulfhydryl interaction which results in suppression of activity when Ca^{++} or K^+ is present as activator. The characteristic properties of EDTA further suggest that the unidentified group interacting with the SH groups is a metal. In a recent note Friess *et al.* (14) have concluded that, if a metal is involved in the EDTA activation, this metal must be very firmly bound and that it is not removed by treatment with EDTA. Analysis of EDTA-treated myosin leads them to suggest that this metal may be Mg. The observations presented here further suggest that the groups on the protein involved in this binding of metal are, at least in part, sulfhydryl groups.

SUMMARY

1. The behavior of myosin ATPase after treatment with *p*-chloromercuribenzoate or *N*-ethylmaleimide has been studied with either Ca^{++} or ethylenediaminetetraacetic acid as activator.

2. The ATPase activity of myosin exhibited a marked increase with Ca^{++} as activator when about one-half the sulfhydryl groups had been titrated with PCMB. Further titrations led to inhibition, the process being complete when all the SH groups had reacted.

3. On the other hand, only inhibition was observed with EDTA as activator, the results indicating that removal of only one-half of the SH groups is required for complete inactivation when EDTA is used as activator.

4. The results with NEM were qualitatively similar. With EDTA as activator, binding of no more than one-quarter of the SH is required for complete inactivation.

5. In attempting to reverse the effects of PCMB with β-mercaptoethanol, only the activity as measured in the presence of EDTA appeared to follow a course which is the reverse of PCMB combination, starting at any degree of PCMB combination.

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INDUCTION AND PURIFICATION OF α- AND β-HYDROXYSTEROID DEHYDROGENASES*

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Metabolic interconversions of hydroxy- and ketosteroids have been recognized in a variety of biological systems (1, 2). The enzymatic mechanisms of such interconversions have become clarified by the isolation of two bacterial enzymes catalyzing reversible oxidations of certain 3- and 17-hydroxysteroids. These enzymes have been obtained in a state of sufficient purity to characterize their coenzyme requirements, substrate specificity, and reaction kinetics (3–6).

Pseudomonas testosteroni is a microorganism capable of utilizing C-19 steroids as its sole source of organic carbon (3). Growth in a culture medium containing testosterone or similar steroids causes the induction of two soluble DPN[1]-linked hydroxysteroid dehydrogenases. The reactions (I,
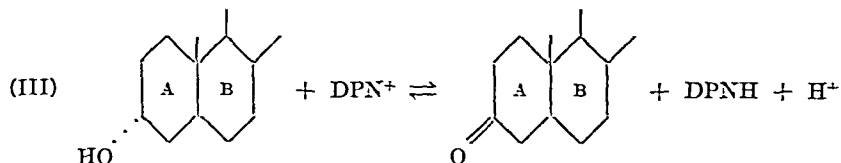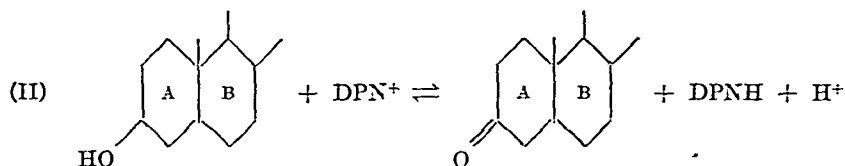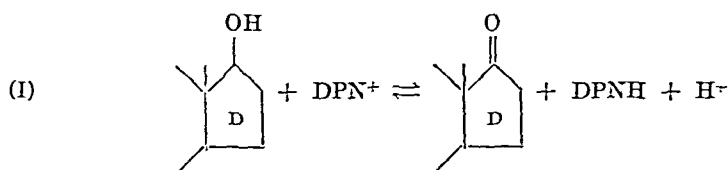
(I) $+ DPN^+ \rightleftharpoons$ $+ DPNH + H^+$

(II) $+ DPN^+ \rightleftharpoons$ $+ DPNH + H^+$

(III) $+ DPN^+ \rightleftharpoons$ $+ DPNH + H^+$

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[1] The following abbreviations are used: DPN = diphosphopyridine nucleotide; DPN⁺ = oxidized diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide; EDTA = disodium ethylenediaminetetraacetate (Versene).

II, and III) catalyzed by these enzymes may be represented by the accompanying partial formulae.

Reactions of type (I) and (II) are catalyzed by a single apoenzyme, β-hydroxysteroid dehydrogenase (designated here as β enzyme), whereas those of type (III) are catalyzed by another enzyme, α-hydroxysteroid dehydrogenase (designated here as α enzyme). The A:B ring fusion may be either cis or trans in reactions (I), (II), and (III). The present study is concerned with the conditions required for the induction of these enzymes as well as improved methods for their separation and purification. The complete separation of these enzymes is of importance for the enzymatic microassay of certain functional groups on steroids (4, 7) and for this reason individual protocols for purifications are given in some detail.

Both α and β enzymes apparently require free sulfhydryl groups for activity and are quite labile, especially in the presence of heavy metal ions (4, 5). α enzyme is more sensitive in this respect than the β enzyme, and both enzymes are stabilized by their coenzyme (DPN$^+$). The incorporation of DPN$^+$ during the purification of α enzyme (5) was rendered unnecessary when vigorous efforts were made to exclude heavy metal contaminants. Application of rapid, direct spectrophotometric methods for the assay of enzyme activities greatly facilitated the purification procedures.

<div align="center">EXPERIMENTAL</div>

<div align="center">Materials and Methods</div>

Microorganism—*P. testosteroni* (ATCC 11996), which is capable of utilizing testosterone and related steroids as sole source of carbon (*cf.* (3)), was maintained by weekly subculture on 2 per cent agar slants containing 0.05 per cent testosterone in an otherwise completely inorganic medium. This microorganism was isolated by enrichment culture from soil obtained in Berkeley, California. The characteristics of this microorganism have not been previously described.[2] It is a highly motile, non-pigmented, gram-negative, straight rod with a single polar flagellum and does not form spores. It is a strict aerobe, grows on C-19 steroids, acetate, lactate, yeast extract, or casein hydrolysates. Growth is optimal at about 30°, but is very sparse at 37°.

Growth Medium—Bacterial cells were grown in a liquid medium composed of 1.0 gm. of $(NH_4)H_2PO_4$, 1.0 gm. of $(NH_4)_2HPO_4$, 2.0 gm. of KH_2PO_4, and 10.0 ml. of trace elements[3] per liter of distilled water. To

[2] We are indebted to Dr. R. S. Benham, Department of Medicine, University of Chicago, for this identification.

[3] The trace element solution had the following composition: 20 gm. of $MgSO_4 \cdot 7H_2O$, 1 gm. of NaCl, 0.5 gm. of $FeSO_4 \cdot 7H_2O$, 0.5 gm. of $ZnSO_4 \cdot 7H_2O$, 0.5 gm. of $MnSO_4 \cdot 3H_2O$, 0.05 gm. of $CuSO_4 \cdot 5H_2O$, and 10 ml. of 0.1 N H_2SO_4 per liter.

this mineral base yeast extract (Difco) was added to give a final concentration of usually 10.0 gm. per liter and the medium was autoclaved. The steroid was dispersed separately in a small volume of water by treatment in a 9 kc. 50 watt Raytheon sonic oscillator for 30 minutes. This treatment produced a sterile, fine suspension which was added to the remainder of the autoclaved medium just prior to inoculation.

Reagents—Analytical reagent grade ammonium sulfate was recrystallized at least twice from EDTA (2.0 gm. per liter) made alkaline to pH 8.0 with NH₄OH (8). Commercial protamine sulfate (Eli Lilly and Company) was purified by dissolving in EDTA (2 gm. per liter) and reprecipitating with ethanol. This procedure was carried out twice and the resultant viscous mass was washed with ethanol and dried *in vacuo*. DPN⁺ of 85 per cent purity on a weight basis was obtained from the Pabst Laboratories, Milwaukee, Wisconsin. All steroids were carefully purified by crystallization and sublimation until melting point and specific rotation agreed with figures given in the literature.

Determinations—The enzyme assays were carried out spectrophotometrically with testosterone as substrate for β enzyme and androsterone for α enzyme. The reaction system contained 100 μmoles of sodium pyrophosphate buffer (1.0 ml. of 0.1 M, pH 8.9), 0.5 μmole of DPN⁺, 15 γ of steroid, and 0.02 to 0.1 ml. of enzyme in a total volume of 3.0 ml. and had a final pH of 9.1. The reactions were carried out in 10 mm. light path cuvettes in a Beckman model DU spectrophotometer equipped with thermospacers for temperature regulation to 25.0° ± 0.1°. The reactions were initiated by the addition of enzyme, and optical density measurements were taken at 340 mμ against a control cell containing all components except steroid. Readings were taken 30 seconds after enzyme addition and usually every 15 seconds thereafter for 2 to 3 minutes. Velocities were calculated from the slopes, utilizing the zero order portion of optical density *versus* time graphs. 1 unit of either α or β enzyme was defined as causing an optical density change of $\log_{10} I_0/I = 0.001$ per minute under the above conditions.

Protein concentrations were determined during the initial stages (Steps 1 to 3) by a turbidimetric method (9) and later by measurement of light absorptions at 280 and 260 mμ (10). Agreement between these methods was satisfactory.

Enzyme Induction and Extraction

Inducing Substrates—Extracts prepared from cells grown on a medium containing yeast extract but no added steroidal substrates had specific activities for either α or β enzyme varying between 10 and 40 units per mg. of protein. Addition of certain steroids to the growth medium enhanced the specific activities 20 to 100 times. The structural specificity and effi-

ciency of the inductors have not been investigated in detail. Steroids which may serve as carbon sources for the microorganism (3) such as testosterone (4-androsten-17β-ol-3-one), dehydroepiandrosterone (5-androsten-3β-ol-17-one), and 4-androstene-3,17-dione all caused induction of both α and β enzymes (Table I). The relative rates of oxidation of 17β-hydroxyl and 3β-hydroxyl groups were constant in all extracts examined and remained so throughout the purification procedure. The rate of oxidation of 3α-hydroxyl groups varied independently. For these reasons, as well as for others (6), it was concluded that 3β- and 17β-hydroxyl oxidations were

TABLE I

Activities of α and β Enzymes in Extracts of Bacteria Grown on Various Steroids

Steroid in growth medium	Specific activity* of	
	α enzyme	β enzyme
None	40	20
	17	25
Testosterone	1230	667
	1650	560†
	1785	698†
	2220	984†
Dehydroepiandrosterone	3400	880
4-Androstene-3,17-dione	2100	720

* Expressed as units per mg. of protein of extracts prepared by sonic disintegration or by alumina grinding of *Pseudomonas* cells grown in liquid culture on a medium containing 5 gm. per liter of yeast extract and 250 mg. per liter of steroid. Incubated 16 hours at 30°.

† In these experiments, 10 gm. of yeast extract per liter and 500 mg. of steroid per liter were used. The steroid was added after preliminary growth for 16 hours at 30°, and the incubation was continued for 24 hours longer.

associated with the same enzymatic protein, whereas 3α-hydroxyl oxidation was catalyzed by a separate enzyme.

Release of Enzyme by Sonic Disintegration—α and β enzymes remain intracellular and are not released into the culture medium. Grinding of the harvested cell paste with alumina according to the method of McIlwain (11) provided an adequate method of obtaining active enzyme extracts. Cell disintegration in a water-cooled Raytheon sonic oscillator led to more reproducible results and higher yields of enzymes. The *Pseudomonas* cells were readily fragmented by sonic oscillations and 15 minutes treatment resulted in maximal release of enzymes, whereas longer treatment dissolved other cellular fractions, causing the specific activities of the enzymes in the supernatant solution to decline. The enzymes appear to be stable to sonic

treatment in crude extracts for 30 to 40 minutes or possibly even longer, when the temperature of the solution was maintained at 10° or below.

Effect of Steroid Concentration on Enzyme Formation—The influence of the initial steroid concentration on adaptive enzyme formation was studied in a medium containing 5 mg. of yeast extract per ml. and 500, 50, or 5 γ of testosterone per ml. The cultures were incubated for 16 hours at 30° in a platform shaker; the cells were harvested by centrifugation, washed twice with 0.03 M phosphate buffer, pH 7.2, and disintegrated for 15 minutes in the sonic oscillator. After centrifugation for 15 minutes at 10,000 $\times$ g, the clear supernatant liquids were assayed for α and β enzyme activities and protein content. The specific activities of these extracts are recorded

TABLE II

Effect of Concentration of Inducing Steroid on Specific Activities of α and β Enzymes

Testosterone concentration, γ per ml.	Specific activity, units per mg. protein	
	α enzyme	β enzyme
0	10	17
5	58	90
50	168	236
500	370	565

Four cultures were set up, each containing 5 mg. of yeast extract per ml. and varying concentrations of testosterone. Incubated 16 hours at 30°. Cultures were filtered, harvested by centrifugation, washed twice with 0.03 M phosphate buffer, pH 7.2. Disintegrated by sonic treatment for 15 minutes and centrifuged for 15 minutes at 10,000 $\times$ g. Specific activities determined on supernatant solutions.

in Table II. It will be noted that the ratio of the specific activities of α enzyme to β enzyme in these preparations was less than 1, whereas in most extracts the α activity was considerably higher than the β activity.

Age of Culture—Samples were removed from a culture growing on 0.5 mg. of testosterone per ml. and 5.0 mg. of yeast extract per ml. during various stages of growth. The cells were harvested and washed by centrifugation, and extracts were prepared by sonic disintegration. The specific activities of α and β enzymes were determined in these extracts. At successive time intervals, the culture density and dissolved testosterone concentration were measured. The latter was determined enzymatically (4) on the filtered culture medium after removal of cells by centrifugation (Fig. 1). Maximal enzyme yield was reached after the culture density curve indicated the cessation of a logarithmic type growth phase and the onset of the stationary phase.

Initially, the dissolved steroid concentration in the culture medium was

about 25 γ per ml. Between 12 and 18 hours of the growth period the amount of testosterone in solution increased to about 40 γ per ml., indicating the possible production by the growing cells of a compound which raises steroid solubility. In a comparatively short time after this, the concentration of testosterone fell to zero, and the specific enzyme activities
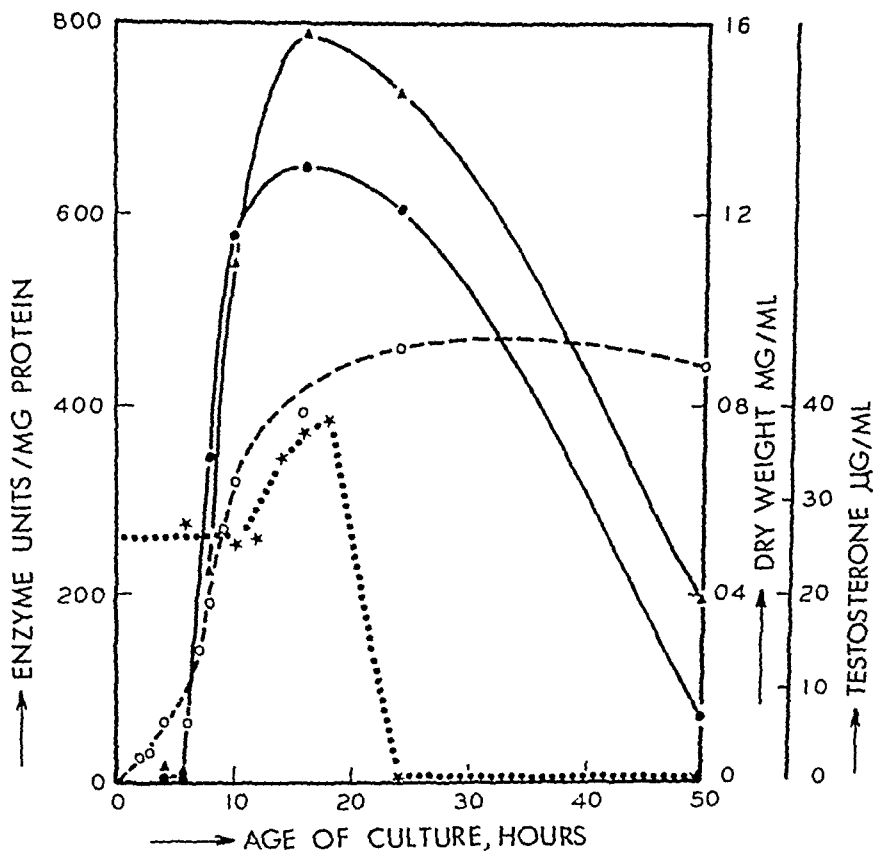
FIG. 1. Relation between specific activities of β (●) and α (▲) enzymes and age of culture. Also shown are the culture density (O) and the dissolved testosterone concentration (★) in the culture medium. Culture grown on 0.5 per cent yeast extract and 0.05 per cent testosterone at 30°. Samples removed at varying times and filtered (to remove undissolved testosterone); cells harvested and washed by centrifugation. 15 minute sonic disintegration, followed by centrifuging for 15 minutes at 20,000 × g. Enzyme activities measured on supernatant fluids.

began to decline beyond this point. Maximal specific activities were reached at about 20 hours, but, since slight growth was still occurring at this point, maximal enzyme production was not achieved. It was found that adaptive enzyme formation was always delayed 6 to 8 hours after the addition of the steroid, even when an adapted inoculum was used. This proved true, whether the steroid was added initially or at any time within 24 hours of inoculation. In order to maximize the yield of the dehydrogenases, delayed addition of the steroid was adopted, whereby the cultures

were grown on yeast extract only for 16 hours, the steroid was then added, and the incubation was prolonged for another 20 to 24 hours.

Purification of Enzymes

A separation of α and β enzymes resulting in negligible contamination of activities and a 50- to 150-fold purification of each enzyme have been achieved by a four-step procedure: Step 1, a first ammonium sulfate fractionation, which resulted in a preliminary separation of α and β enzymes into fractions, each of which was then carried through Steps 2 to 4; Step 2, precipitation of nucleic acids as their protamine salts; Step 3, a second ammonium sulfate fractionation; Step 4, adsorption of extraneous proteins on calcium phosphate gel.

In separating the enzymes advantage is taken of the fact that at 0° β enzyme is precipitated by 0.30 to 0.40 saturated ammonium sulfate, whereas the α enzyme precipitates at higher saturations between 0.40 and 0.55. The method here described was developed from earlier attempts in which large losses of activity were encountered. The success of the procedure depends upon the careful elimination of heavy metals through the use of glass-distilled water (prepared by distillation through a Vigreux column), washing of all glassware with dilute solutions of EDTA (2 gm. per liter), the use of carefully purified ammonium sulfate and protamine sulfate, and the incorporation of EDTA into solutions at a level of 0.001 M at all stages possible. Reproducible and complete separations of the enzymes depend further upon the slow mechanical addition of ammonium sulfate at a uniform rate, while the enzyme solution is being stirred magnetically. The ammonium sulfate additions were performed usually during 2 to 3 hours for a 5 per cent increase in saturation, and precipitates were permitted to accumulate for at least 12 and preferably 24 hours before being separated by centrifugation. A simple device for the mechanical addition of ammonium sulfate has been described (8).

Preparation of Extracts—*Pseudomonas* cells were grown in low form culture flasks (2500 ml. capacity) with a large ratio of bottom surface to volume (Corning Glass Works, No. 4422). Each flask contained 1200 ml. of mineral medium and 10 gm. of Difco yeast extract per liter. The flasks were well agitated on a horizontal platform shaker and the temperature was maintained at 30°. Each liter of the medium was inoculated with 5 ml. of an 8 to 10 hour-old starter culture, and the cultures were permitted to grow for 15 to 16 hours, at which time a testosterone suspension (prepared as described above) was added to make the final concentration of steroid 500 mg. per liter. Incubation was continued for 24 hours longer. The cultures were chilled and then filtered through coarse filter paper on a large Büchner funnel with suction, and the cells were harvested by a

single passage through a Sharples continuous supercentrifuge (25,000 r.p.m.) at a rate of about 300 ml. per minute. In a typical experiment, 14.4 liters of culture fluid (twelve flasks) contained 1.42 mg. dry weight of cells per ml. by optical density measurement. The wet weight of harvested bacteria was 87 gm. These were washed twice by centrifugation with 0.03 M phosphate buffer, pH 7.2, containing 0.001 M EDTA and finally suspended in 200 ml. of the same buffer at a final concentration of 82.0 mg. dry weight per ml. Each 40 ml. aliquot of this suspension was then treated in the sonic oscillator for 15 minutes. All subsequent manipulations were carried out as close to 0° as possible. Cellular débris and large particles were removed by centrifugation for 20 minutes at 20,000 × g. The residue was reextracted with buffer and centrifuged and the clear supernatant solutions were combined. The initial extract had a volume of 260 ml. and specific activities of 2220 units of α enzyme per mg. of protein (range 1200 to 2220) and 984 units of β enzyme per mg. of protein (range 500 to 1000).

Step 1—Solid purified ammonium sulfate was added to the extract in the manner described. The saturation was raised to 30 per cent and then fractions were obtained at 5 per cent increments in concentrations up to 60 per cent. Each fraction was redissolved in 20 to 50 ml. of 0.03 M phosphate, pH 7.2, containing 0.001 M EDTA. The distribution of activities of the two enzymes is shown in Table III. 75 to 85 per cent of the total activities of both enzymes were recovered, provided recrystallized ammonium sulfate was used; otherwise there was a considerable loss of α enzyme. The purification obtained at this stage is of the order of 3- to 4-fold for both enzymes. The 30 to 40 per cent fractions containing 42.6 per cent of the β activity and 11 per cent of the α activity were combined and used for further purification of β enzyme. The 45 to 55 per cent fractions containing 48.3 per cent of the α activity and less than 1 per cent of β contamination were combined for further purification of α enzyme. The 40 to 45 per cent ammonium sulfate fraction showed least separation of the two enzymes and was eliminated. This fraction was usually refractionated according to Step 1.

Step 2—α and β enzyme-containing fractions were then each treated with protamine by the addition of a solution of protamine sulfate (10.0 mg. per ml., pH 6.0). The protamine sulfate was added in excess, which was achieved at a ratio of about 1 part by weight of protamine sulfate to 5 parts of protein. The copious precipitate was permitted to accumulate overnight and removed by centrifugation. The supernatant solution contained all of the β enzyme activity, but about 65 per cent of the α enzyme activity was lost in this step unless purified protamine sulfate was employed. This treatment reduced the viscosities of the solutions and

raised the ratio of the optical density at 280 mμ to that at 260 mμ from about 0.6 to approximately 1.0. Tables IV and V give the activities before and after protamine treatment.

TABLE III

Distribution of α and β Enzyme Activities in Step 1

Per cent saturation of ammonium sulfate	Total activity		Specific activity, units per mg. protein	
	α enzyme, units $\times 10^5$	β enzyme, units $\times 10^5$	α enzyme	β enzyme
	Initial extract			
	13.0	5.73	2220	984
0–30	0.71	1.21	600	1020
30–35	0.26	0.84	1130	3720
35–40	1.17	1.61	1440	2720
40–45	2.50	0.53	4330	929
45–50	3.96	0.05	7270	87
50–55	2.33	0.002	4890	5
55–60	0.17	0.0004	401	1

TABLE IV

Purification Steps 2 and 3 for β Enzyme

Fraction	Total activity		Specific activity, units per mg. protein		Ratio, $\frac{280}{260}$ mμ
	α enzyme, units $\times 10^5$	β enzyme, units $\times 10^5$	α enzyme	β enzyme	
30–40% from Table III*	1.45	2.62	1474	2660	0.58
Step 2. Supernatant from protamine precipitation	1.49	2.56	1940	3340	1.09
Step 3.					
0–25	0.033	0.31	298	2730	
25–30	0.021	0.49	287	6960	
30–35	0.104	1.09	510	5350	
35–40	0.175	0.25	1730	2440	
Supernatant	0.762	0.021	7630	215	

* These figures represent new assays on combined fractions and therefore agree with those in Table III only within the experimental error.

Step 3—The supernatant fluids from the protamine precipitation were then subjected to further ammonium sulfate fractionation by the technique already described. The precipitates were dissolved in 0.01 M phosphate, pH 7.2, containing 0.001 M EDTA. The major activity and purest

fractions of β enzyme were precipitated between 25 and 35 per cent saturation (Table IV), whereas α enzyme was found in the 40 to 50 per cent ammonium sulfate fractions (Table V). The specific activities of both enzymes at this stage ranged between 5000 and 13,000 units per mg. of protein in several purification procedures. The contamination of α enzyme by β enzyme is negligible (Table V). Unless highly purified ammonium sulfate is used at this stage, the major portion of α activity is lost. With the highly purified ammonium sulfate, contamination of β by α enzyme at this stage may amount to about 5 to 8 per cent (Table IV). This contamination is reduced by washing the protein precipitates with 40 per cent ammonium sulfate solution. The fractions are stable for at

TABLE V

Purification Steps 2 and 3 for α Enzyme

Fraction	Total activity		Specific activity, units per mg. protein	
	α enzyme, units $\times 10^5$	β enzyme, units	α enzyme	β enzyme
45–55% from Table III*	6.65	39,000	5380	32
Step 2. Supernatant from protamine precipitation	6.15	27,000	6720	32
Step 3.				
0–40	1.26	25,200	4350	870
40–45	2.44	7,480	9210	28
45–50	1.83	2,490	9330	13
50–55	0.52	260	4520	3

* These figures represent new assays on combined fractions and therefore agree with those in Table III only within the experimental error.

least 1 year in the frozen state at this stage and have usually been taken individually through Step 4.

Step 4—The majority of gel purification procedures involve an initial enzyme adsorption and a subsequent elution; more nearly ideal conditions are met in a one-step procedure in which a preferential adsorption of substantial amounts of other proteins leaves the major portion of the desired enzymes in solution. Such a preferential adsorption with calcium phosphate gel has been used to secure an additional 5- to 10-fold purification of α and β enzymes obtained from Step 3.

Enzyme extracts secured prior to the second $(NH_4)_2SO_4$ fractionation have consistently given less satisfactory purifications with gel adsorptions (2- to 4-fold), whereas enzyme extracts treated with gel prior to removal of nucleic acids by protamine sulfate precipitation gave little or no purifica-

tion by this method. Likewise, repeated gel adsorptions resulted in only small additional degrees of purification.

Gel adsorption properties are known to depend upon methods of preparation, conditions of storage, pH, ionic strength, age, and some still undetermined factors. Calcium phosphate gel was prepared by the method of Keilin and Hartree (12). The pH was adjusted with dilute acetic acid to 6.5, and, after thorough washing by centrifugation, the gel was suspended in double distilled water to give a concentration of approximately 15 mg. dry weight of gel per ml. The gel suspensions were stored at 2–4° and used within 1 or 2 days, or at most 1 week from the time of preparation. Gels stored for longer periods of time were found to have unpredictable adsorption characteristics.

TABLE VI

Effect of Calcium Phosphate Gel Adsorption on α and β Enzyme Purification

Enzyme	Material	Total protein	Total activity	Specific activity, units per mg. protein
		mg.	*units*	
α	Extract from Step 3	11.0	77,500	7,800
	Supernatant after calcium phosphate gel adsorption	1.0	27,000	57,000
β	Extract from Step 3	36.0	960,000	13,800
	Supernatant after calcium phosphate gel adsorption	5.5	480,000	90,000

Calcium phosphate gel (10 to 20 mg. per ml.) prepared the previous day is added to the protein solution (2 to 10 mg. per ml.), obtained from Step 3, in the optimal gel to protein ratio. After a 5 to 10 minute adsorption period and 5 minutes of centrifuging at about 2000 × g, the supernatant liquid was removed and assayed for enzyme activity and protein content.

The critical gel to protein ratio varies from about 5 to 20 for α and β enzymes, when expressed as mg. dry weight of gel to mg. of protein. Both enzymes may be adsorbed completely on the gel if sufficiently large quantities of gel are used, and, if insufficient gel is employed, the purifications are inadequate. The optimal gel to protein ratio is best determined by a small scale pilot experiment. Under appropriate conditions approximately 60 to 90 per cent of the inactive proteins is adsorbed, leaving most of the enzyme in solution. Table VI illustrates purifications of α and β enzymes obtained by calcium phosphate gel adsorption and represents the enzyme preparations used in specificity and kinetic studies (6). Specific

activities of 50,000 to 100,000 units per mg. of protein for both enzymes have been obtained routinely. These preparations have been stored at −20° for at least 1 year without loss in activity, but slowly inactivate in dilute solutions at 0°.

Purity of Preparations—Specific activities of 50,000 to 100,000 units per mg. of protein represent turnover numbers of 2500 to 5000 per minute at 25°, assuming a molecular weight of 100,000 for each enzyme. The purifications obtained are of the order of 50- to 150-fold over the adapted extracts, but, since the specific activities of these enzymes are enhanced 20 to 100 times by adaptation to specific substrates, this degree of purification is not strictly comparable with non-adaptive enzymes.
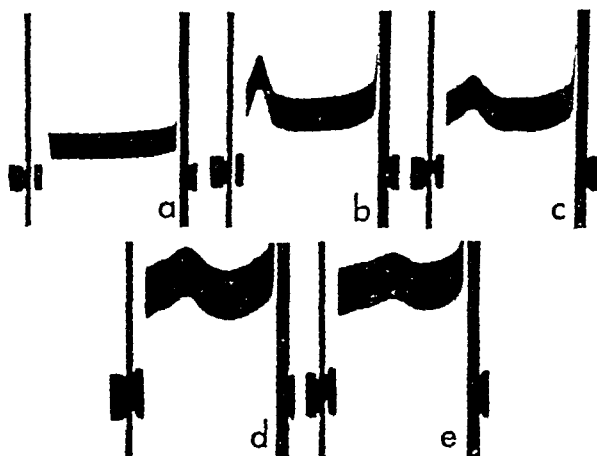
FIG. 2. Sedimentation velocity patterns of α enzyme in 0.1 M NaCl, pH 7.0, observed in a cell of 12 mm. optical path proceeding to the right at 32 minute intervals. Rated speed, 59,780 r.p.m.; average rotor temperature, 13.3°. Protein concentration, 5.0 mg. per ml. The enzyme had a specific activity of 59,800 units per mg. of protein. $s_{20,w} = 3.35 \times 10^{-13}$ second.

No extensive criteria for homogeneity have so far been obtained. Both preparations are free from ethanol dehydrogenase but are contaminated by a distinct steroid isomerase (13) which catalyzes the conversion of 5-androstene-3,17-dione to 4-androstene-3,17-dione. α enzyme preparations contain much more of this isomerase than β enzyme preparations. An ultracentrifugal sedimentation pattern of an α enzyme preparation (Fig. 2) demonstrates a single major peak with small amounts of both a slower and a faster moving component. The sedimentation constant for the major peak (presumably α enzyme) is $s_{20,w} = 3.35 \times 10^{-13}$ second in 0.1 M NaCl, 0.001 M EDTA, 0.003 M NaHCO₃, pH 7.0.

DISCUSSION

Small amounts of α and β enzymes (of the order of 1 to 5 per cent of the maximal adapted specific activities) are present in cells grown on yeast

extract as carbon source. Since yeasts are known to contain sterols such as ergosterol, and yeast extract gives a Liebermann-Burchard reaction, the possibility that such compounds may act as inducing substrates in cells grown on yeast extract cannot be excluded. When steroids having either ketone or β-hydroxyl groups at positions 3 or 17 are added to the growth medium, induction of both α and β enzymes occurs, whereas none of these compounds is a substrate for the α enzyme-catalyzed reaction. 3α- and 3β-hydroxysteroids are geometrical isomers. The induction of an enzyme specific for a certain geometrical configuration by compounds having either the requisite or opposite configuration has been reported by Monod, Cohen-Bazire, and Cohn (14), who showed that the formation of β-galactosidase in *Escherichia coli* was highly specific for inductors with an intact galactosidic radical, but that such inductors were not necessarily substrates for the enzyme. In particular, α-galactosides acted as inductors, although they are not hydrolyzed by β-galactosidase. The α- and β-hydroxysteroid dehydrogenase induction is understandable in terms of the intracellular formation of a common intermediate which may act as the inductor. 4-Androstene-3,17-dione or androstane-3,17-dione may possibly be such an intermediate.

SUMMARY

Cell-free extracts of a *Pseudomonas* species contain two adaptive DPN-linked steroid dehydrogenases: α-hydroxysteroid dehydrogenase which catalyzes the reversible oxidation of 3α-hydroxysteroids to the corresponding ketosteroids and β-hydroxysteroid dehydrogenase which catalyzes the oxidation of 3β- and 17β-hydroxysteroids to the respective ketosteroids. Both enzymes are induced in response to the addition to the growth medium of single steroids which need not bear α or β oriented hydroxyl groups. Separation of the enzymes and purification to the extent of 50- to 150-fold over their adapted levels have been achieved by a four-step procedure.

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SPECIFICITY, KINETICS, AND INHIBITION OF α- AND β-HYDROXYSTEROID DEHYDROGENASES*

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Two adaptive bacterial hydroxysteroid dehydrogenases have been separated and obtained as highly purified proteins (1). These enzymes are (1) a β-hydroxysteroid dehydrogenase (designated here as β enzyme) which catalyzes the reversible oxidations of 3β- and 17β-hydroxysteroids by DPN^+[1] and (2) a 3α-hydroxysteroid dehydrogenase (designated as α enzyme) which catalyzes reversible DPN^+-linked oxidations of 3α-hydroxysteroids. These enzymes are characterized by remarkably high affinity and structural specificity for their substrates. A detailed study of the reaction kinetics of β enzyme with a variety of related steroids has given information on the structural factors involved in binding of steroids to this enzyme surface and has led to speculation on the possible influence of such factors on the physiological activities of steroids (2).

The present experiments, carried out with highly purified preparations of α and β enzymes, deal with certain aspects of their substrate and coenzyme specificity. The velocities and equilibria of reactions catalyzed by these enzymes will be considered. The freely reversible nature of the reactions studied and the simple manner by which they may be made complete in either direction have made these enzymes useful as preparative and analytical tools. Initial demonstrations of the enzymatic microassay and identification of steroids (3, 4) have now been extended. The stereospecific nature of the reactions catalyzed by these enzymes may be used to advantage in the selective reduction of 3-ketosteroids to yield either 3α- or 3β-hydroxysteroids by the use of α or β enzymes, respectively. Moreover, reduction of a 17-ketosteroid by DPNH with β enzyme yields the 17β-hydroxy isomer only.

By virtue of their high substrate affinity and specificity, the hydroxysteroid dehydrogenases represent ideal models for the study of the interaction of steroids with protein surfaces and the influence of structurally related inhibitors on this process. It will be shown that 1,3,5-estratrien-

[1] The following abbreviations are used: DPN^+ = oxidized diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide.

3-ol and derivatives, as well as the synthetic estrogens diethylstilbestrol and diethylhexestrol, are powerful inhibitors of β enzyme.

Substrate Specificity of α and β Enzymes—The substrate specificities of these enzymes have been partially analyzed (2–4). β enzyme has been found to oxidize 3β- and 17β-hydroxysteroids, as well as 16β-hydroxysteroids although at a slower rate. The product in each case is the corresponding ketone. Oxidation of secondary 17β-hydroxyl groups occurs in compounds *with or without an oxygenated function at position 3*. The presence of additional oxygenated substituents at other points on the steroid skeleton such as in 4-androstene-11α,17β-diol-3-one, 4-androstene-11β,17β-diol-3-one, or 4-androstene-6β,17β-diol-3-one does not prevent the oxidation of the 17β-hydroxyl group. However, in order for 17β-OH or 16β-OH oxidation to occur, the adjacent position (*i.e.* 16 or 17) must be devoid of an oxygenated function. The configuration of the A:B ring fusion is not material to the specificity, since either androstane or etiocholane derivatives participate in the reaction. Moreover, compounds with aromatic A rings such as estradiol-3,17β, estradiol-3,16β, or 1,3,5-estratrien-17β-ol are also substrates for β enzyme.

Oxidation at position 3 of the steroid nucleus by β enzyme occurs only if the secondary alcohol group is β oriented. 3β-Hydroxysteroids of the androstane, etiocholane, 4-androstene, or 5-androstene series are all oxidized. An oxygenated function at position 17 is not essential for the oxidation of the 3β-hydroxyl group. The presence of a short side chain at C_{17}, as in 17α-methyl-5-androstene-3β,17β-diol and 5-pregnen-3β-ol-17-one, does not prevent the oxidation of the 3β-hydroxyl group, but cholesterol with its longer side chain is not oxidized. Specific information has been obtained that the following hydroxyl groups are not oxidized by β enzyme: 3α, 6β, 11α, 11β, 16α, 17α, 20α, and 21.

α enzyme oxidizes only 3α-hydroxysteroids with androstane, etiocholane, cholane, and pregnane type skeletons; however, a substituent at C_{17} is not required as androstan-3α-ol is oxidized. No oxidations by α enzyme of the following hydroxyl groups have been noted: 3β, 6β, 11α, 11β, 17α, 17β, 16α, 16β, 20α, or 21.

Velocities of Oxidations—A detailed analysis of the relation between oxidation velocity and substrate concentration has been carried out for β enzyme (2). Many of the substrates studied, *e.g.* testosterone (see Fig. 1), exhibit kinetics inconsistent with the simple postulates of Michaelis and Menten (5), since the velocity of oxidation shows a marked tendency to decline at high substrate concentrations. In other cases, such as estradiol-3,17β, no inhibition of velocity at high substrate concentrations was

observed (Fig. 1). The kinetics of velocity inhibition at high substrate concentrations are in accordance with the theory of the formation of an inactive bimolecular complex between 2 molecules of substrate and each active center on the enzyme (6, 2). Such a theory predicts a bell-shaped curve when velocity is plotted against logarithm of substrate concentration. This curve is symmetrical about the substrate concentration (S_0) at which the maximal observed velocity (V_0) obtains. S_0 for testosterone at
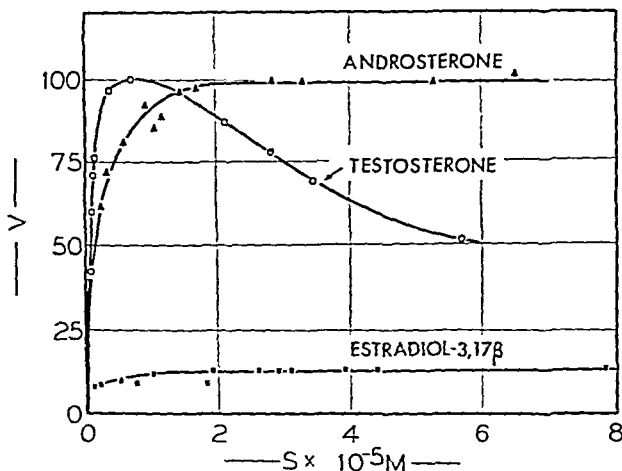
Fig. 1. Velocity of oxidation (V) as a function of substrate concentration (S). Measurements for testosterone and estradiol-3,17β were obtained with β enzyme and velocities were expressed relative to the maximal velocity of oxidation of testosterone (100) at $S = 6.0 \times 10^{-6}$ M. The velocities for androsterone are calculated relative to the maximum (100) obtained from a Lineweaver-Burk plot. Velocities were measured at pH 9.1 in 0.03 M pyrophosphate buffer at 25° with an initial DPN+ concentration of 1.7×10^{-4} M. Initial rates of reaction were obtained from measurements of optical density (340 mμ) against time. For low substrate concentrations reactions were carried out in 10.0 cm. light path cells.

25° and pH 9.1 is 6.0×10^{-6} M in pyrophosphate buffer. V_0 for testosterone is arbitrarily set at 100 and all velocities in Table I are expressed relative to these conditions. In those cases in which no significant inhibition at high substrate concentrations was observed, the kinetics have been analyzed on the basis of the Michaelis and Menten theory (5) and the maximal oxidation velocity (V_m) was obtained according to the graphical solution of Lineweaver and Burk (7).

The velocity of oxidation of androsterone by α enzyme as a function of substrate concentration is also shown in Fig. 1. The Michaelis constant is 1.6×10^{-6} M at pH 9.1 and 25°, and over a wide range of androsterone concentrations there is no inhibition by excess substrate. Table II gives

the relative rates of oxidation of various C-19, C-21, and C-24 3α-hydroxysteroids by α enzyme.

Influence of pH on Equilibrium and Velocity—The profound effect of hydrogen ion concentration on the equilibria of reactions catalyzed by enzymes requiring DPN+ has been pointed out for alcohol dehydrogenase (8, 9). Since hydrogen ions participate in the reactions, the pH markedly affects the equilibria. In the presence of large amounts of enzymes, measurements were made at 25° and at different pH in a system of the fol-

TABLE I

Maximal Relative Rates of Enzymatic Oxidation (V_0 or V_m) of 3β- and 17β-Hydroxysteroids by β Enzyme at Optimal Substrate Concentrations

Compound	V_0 or V'_m	S_0, $\times 10^{-6}$ M
Testosterone	100	6.5
19-Nortestosterone	80.0	6.7
6β-Hydroxytestosterone	96	6.0
11α-Hydroxytestosterone	61.7	*
11β-Hydroxytestosterone	24.4	*
Androstan-17β-ol-3-one	90	3.0
Androstan-3β-ol-17-one	74.6	4.0
5-Androsten-3β-ol-17-one	17.4	10.5
Androstanol-3β	44.0	*
Etiocholan-17β-ol-3-one	84.7	35.0
Etiocholan-3β-ol-17-one	74.6	16.0
Estradiol-3,17β	13.3	*
1,3,5-Estratrien-17β-ol	134	31.0
Estradiol-3,16β†	2.3	*

* Indicates lack of significant velocity inhibition by excess substrate and consequent non-applicability of S_0 term. In these cases V_m was obtained from plots according to Lineweaver and Burk.

† 16β-OH is the only other position found to be oxidized by β enzyme.

lowing composition: 100 μmoles of buffer (either orthophosphate or pyrophosphate), 0.5 μmole of DPN+, and 0.1 μmole of steroid (androsterone or testosterone). From the total change in optical density at 340 mμ, the amount of DPNH formed was calculated ($\epsilon = 6220$). Assuming that for each mole of steroid oxidized 1 mole of DPN+ was reduced, equilibrium constants were calculated according to the following equations (9):

$$(1) \qquad K_H = \frac{(\text{androstane-3,17-dione})(\text{DPNH})(\text{H}^+)}{(\text{androsterone})(\text{DPN}^+)}$$

$$(2) \qquad K_H = \frac{(\text{4-androstene-3,17-dione})(\text{DPNH})(\text{H}^+)}{(\text{testosterone})(\text{DPN}^+)}$$

These equilibrium constants are independent of pH, and the mean value of K_H for reaction (1) was found to be 5.8×10^{-9} and that for reaction (2) 2.6×10^{-8}. The latter figure is a revision of a preliminary value of 3.6×10^{-8} (3) which was obtained with a much less pure enzyme preparation, which is now known to have been contaminated by α enzyme. It has been pointed out that equilibrium measurements may vary considerably with purity of enzyme (9). If the equilibrium constants are calculated without considering the hydrogen ion concentration, one may derive apparent equilibrium constants which are pH-dependent and which yield a linear

TABLE II

Relative Rates of Enzymatic Oxidation of 3α-Hydroxysteroids at Concentrations of about 2×10^{-5} M

Steroid	Rate of oxidation[*]
Androstan-3α-ol-17-one (androsterone).................	100
Androstane-3α,17β-diol.................................	100
Etiocholan-3α-ol-17-one................................	100
Etiocholane-3α,11β-diol-17-one........................	10
Pregnane-3α,17α-diol-11,20-dione-21-acetate (tetrahydro Compound E acetate).................................	10
Deoxycholic acid......................................	10
Androstan-3α-ol.......................................	2

3.0 ml. system containing 100 μmoles of pyrophosphate buffer, pH 9.1, 0.6 μmole of DPN$^+$, 0.06 μmole of steroid in 0.1 ml. of CH_3OH, and 1 to 5 γ of enzyme protein. The measurements were made at 25° and at 340 mμ in 1 cm. light path cells against control cells containing all components except steroid. The velocities were calculated from zero order curves.

[*] Relative to V_0 for androsterone arbitrarily set at 100.

plot of unit slope when plotted logarithmically against pH (Fig. 2). This indicates that the position of the equilibrium of both reactions is directly proportional to the H^+ concentration, and therefore shifted by a factor of 10 for each unit change in pH. The standard free energy changes calculated from the equilibrium constants are -427 kilocalories per mole for the conversion of testosterone to 4-androstene-3,17-dione and -465 kilocalories per mole for the oxidation of androsterone to androstane-3,17-dione.

The initial velocities of the forward and backward reactions are dependent upon the pH of the medium. The steroid is most rapidly oxidized at high pH and most rapidly reduced at low pH values. The initial forward and backward velocities for the interconversion of androsterone and androstane-3,17-dione are shown in Fig. 3 as a function of pH, with-

in the range of about pH 6 to 10, for which measurements are practicable because of enzyme and coenzyme stabilities. It is of interest that the logarithms of the velocities are linear functions of pH, for both forward
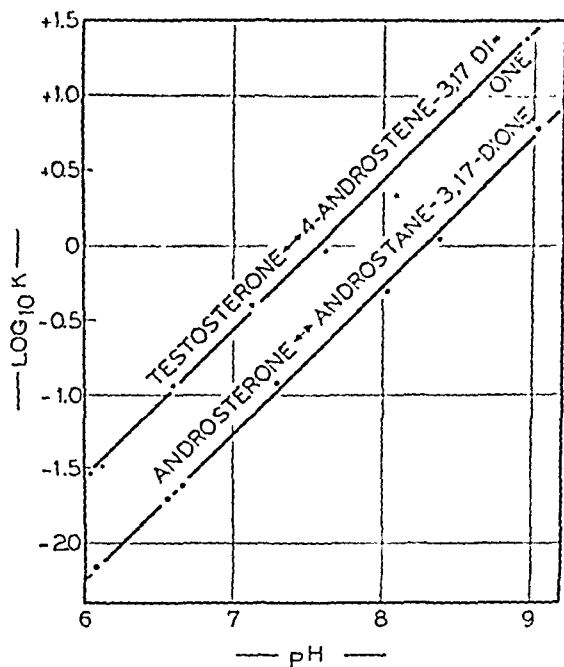
FIG. 2.
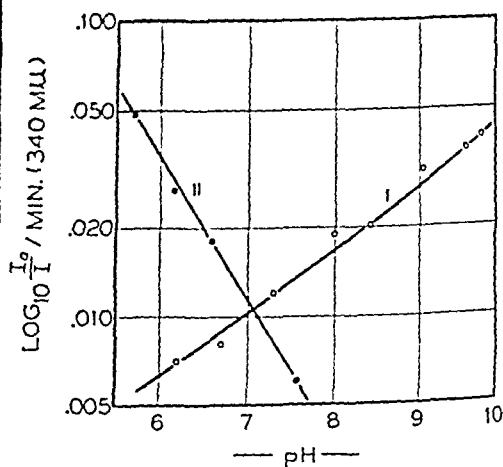
FIG. 3.

FIG. 2. Logarithms of equilibrium constants as a function of pH. The equilibrium constants for the conversion of testosterone to 4-androstene-3,17-dione by β enzyme and androsterone to androstane-3,17-dione by α enzyme were calculated without consideration of the hydrogen ion concentration (see the text). The reaction systems contained, in a 3.0 ml. volume, 100 μmoles of orthophosphate or pyrophosphate buffer, 0.5 μmole of DPN+, 0.1 μmole of testosterone or androsterone in 0.1 ml. of CH_3OH and excess enzyme so that equilibrium was attained in less than 1 minute. Optical density differences were measured at 340 mμ against control cells containing all components except steroid. Temperature 25°.

FIG. 3. Effect of pH on the velocity of interconversion of androsterone and androstane-3,17-dione by α enzyme at 25°. Reaction I was run in a system of 3.0 ml. volume containing 100 μmoles of buffer (orthophosphate or pyrophosphate), 0.5 μmole of DPN+, 20 γ of androsterone in 0.1 ml. of CH_3OH and 0.02 ml. of α enzyme (1400 units per ml.; 5400 units per mg. of protein) which was used to initiate the reaction. Reaction II was run in a system of 3.0 ml. volume containing 100 μmoles of orthophosphate buffer, 0.25 μmole of DPNH, 25 γ of androstane-3,17-dione in 0.1 ml. of CH_3OH and 0.02 ml. of α enzyme which was used to initiate the reaction.

and backward reactions, and thus obey relationships of log $V = k \cdot pH + C$ where k and C are constants. The forward and backward velocities are equal at approximately pH 7.0. The value of k for the forward reaction is +0.70 and for the backward reaction $k = -1.6$.

Enzymatic Assay and Identification of Steroids—The high degree of stereospecificity exhibited by α and β enzymes makes them ideal reagents

for the identification of certain steroid groupings. The simple application of mass action considerations to drive the reaction to completion in either direction increases the usefulness of these enzymes in the microassay of steroids. Thus, with the equilibrium constants calculated for the β enzyme it may be shown that the oxidation of 0.1 μmole of testosterone in a 3.0 ml. system containing 0.5 μmole of DPN+ at pH 9.0 is 98 per cent complete. Conversely, at pH 6.0, the reduction of 0.1 μmole of 4-androstene-3,17-dione to testosterone in the presence of 0.5 μmole of DPNH is 99 per cent complete. The enzyme reduces only the C_{17}-ketone grouping and does not affect the α,β-unsaturated ketone at C_3.

Thus by using an excess of oxidized or reduced coenzyme and appropriate pH the reaction may be made virtually complete for oxidation or reduction. This forms the quantitative basis for the enzymatic microassay of steroids.

Examples of this type of assay with β enzyme were given in an earlier paper (3) for the detection of 17β-OH groups or 17 keto groups. The combined use of α and β enzymes makes it possible to identify and selectively oxidize or reduce certain other groupings.

Fig. 4 shows the assay and identification of two isomers, androstane-3α,17β-diol and androstane-3β,17α-diol, by successive treatment with α and β enzymes in the presence of DPN+. The final product in the first case was androstane-3,17-dione and in the second instance androstane-17α-ol-3-one. Conversely, androstane-3,17-dione may be reduced by DPNH to androstane-3β,17β-diol, androstan-3α-ol-17-one, or androstane-3α,17β-diol by the appropriate use of these enzymes (Fig. 5). The products of the reaction have been inferred by the stoichiometry of reduction of DPN+.

The sensitivity of these assay methods depends upon the volume and light path of the cells and sensitivity of the spectrophotometer. In a system of 0.6 ml. capacity and 1 cm. light path the measurement of 1.0 γ of steroid is easily achieved with a Beckman DU spectrophotometer (Fig. 6).

Michaelis Constants for DPN+—DPN+ is the obligatory hydrogen acceptor for both α and β enzymes. Triphosphopyridine nucleotide is completely inactive when tested in the oxidation of testosterone and epiandrosterone by β enzyme and in the oxidation of androsterone by α enzyme. The affinities of α and β enzymes for DPN+ were measured with several substrates. In each case, the steroid concentration was selected such as to give maximal velocity in the presence of excess coenzyme, and the DPN+ concentration was then varied. The relationship between velocity and DPN+ concentration was found to accord with Michaelis-Menten predictions, and the Michaelis constants were obtained from Lineweaver-Burk plots.

The values for the affinity constants for DPN+ are shown in Table III.

The affinities for DPN+ are considerably lower than those for the steroids (2) but vary greatly with the type of steroid substrate. From the available data it would appear that there is an inverse relationship between the affinity for steroid and for DPN+ under the conditions studied. The interrelationship of the binding properties of steroid and DPN+ to the enzyme
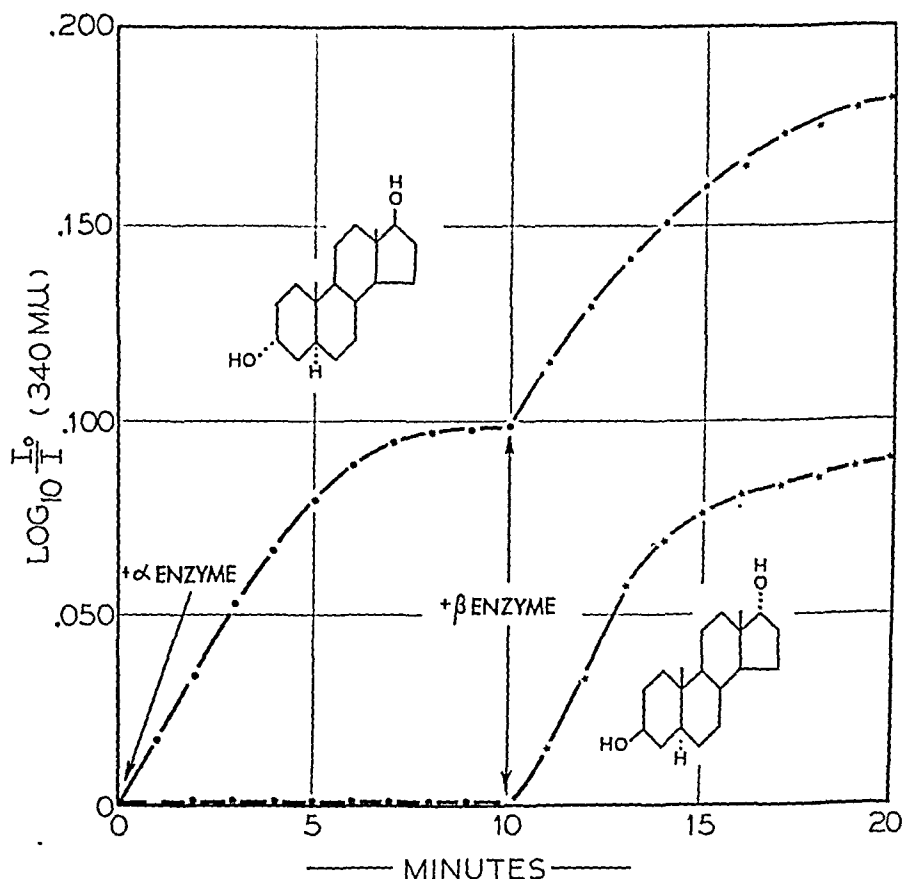
FIG. 4. Assay and identification of androstane-3α,17β-diol and androstane-3β,17α-diol. Optical density measurements at 340 mμ were made with time in 3.0 ml. systems containing 100 μmoles of pyrophosphate buffer, pH 9.1, 0.5 μmole of DPN+, approximately 0.045 μmole of steroid in 0.1 ml. of CH_3OH against controls containing all ingredients except steroid. At zero time, an excess of α enzyme was added to all cuvettes, and, at 10 minutes, an excess of β enzyme was added to all cuvettes.

surface may perhaps be related to the fact that these compounds must occupy during the enzyme reaction positions adjacent on the enzyme surface in order for the direct stereospecific transfer of hydrogen to occur (10). It seems reasonable that the residual secondary valence forces on the enzyme surface must be satisfied by steroid and DPN+ at the time of binding for enzyme action. In those cases in which the steroid is firmly bound, e.g. testosterone, the coenzyme exhibits relatively low affinity, whereas for the

etiocholane derivatives, which are relatively poorly bound to the enzyme surface (2), the affinities for DPN+ are very much higher.

Inhibition of α and β Enzyme Activities by 1,3,5-Estratriene Derivatives— Preliminary experiments indicated that structurally related compounds competed for the enzyme surface and could inhibit the oxidation of testosterone by β-hydroxysteroid dehydrogenase (3). The degree of inhibition depended upon concentration of both substrate and inhibitor, but the
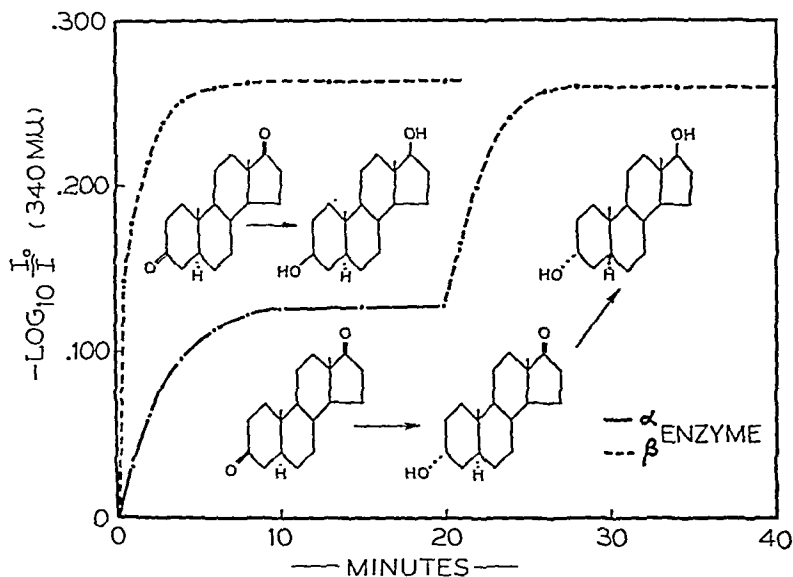
FIG. 5. Reduction of androstane-3,17-dione by α and β enzymes. Decreases in optical density were measured at 340 mμ in 3.0 ml. systems containing 100 μmoles of orthophosphate buffer, pH 6.0, 0.25 μmole of DPNH, 0.063 μmole of androstane-3,17-dione in 0.1 ml. of CH₃OH. At zero time, α enzyme or β enzyme was added to different cuvettes and measurements were begun against a blank cell containing all components except steroid. At 20 minutes, β enzyme was added to the cell containing α enzyme.

analysis of the type of inhibition was complicated by the fact that testosterone and many other substrates had kinetics characterized by inhibition of velocity at high substrate concentration (see above). It was found that the oxidation of testosterone was inhibited by certain 1,3,5-estratriene derivatives, by diethylstilbestrol and diethylhexestrol at low concentrations comparable to those of the substrate, but not at all by much higher concentrations of phenol (Fig. 7). Estradiol-3,17β, itself a substrate for β-hydroxysteroid dehydrogenase, was considerably more firmly bound by β enzyme than testosterone, as shown by experiments with mixtures of these two substrates.

The inhibition of testosterone oxidation by estradiol-3,17α was studied at a series of concentrations of both substrate and inhibitor.   Fig. 8 shows the inhibition obtained at two substrate concentrations.   This and similar results were analyzed according to concepts of competitive and non-competitive substrate-inhibitor interaction and did not appear to obey either pattern, even when the inhibition at high testosterone concentrations was taken into account.   The 50 per cent inhibition point of reaction velocity was observed at a substrate-inhibitor ratio of 3.1 for 1.74×10^{-5} M testosterone and 2.6 for 3.40×10^{-5} M testosterone.
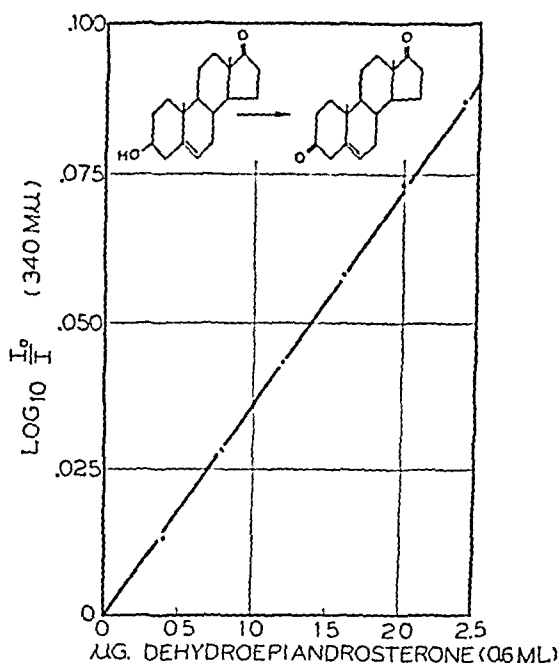
FIG. 6. Microassay of dehydroepiandrosterone by oxidation to 5-androstene-3,17-dione.   The system contained, in a total volume of 0.6 ml., 20 μmoles of pyrophosphate buffer, 0.1 μmole of DPN+, 0.4 to 2.4 γ of dehydroepiandrosterone in 0.02 ml. of CH₃OH and excess β enzyme.   Final pH 9.1; temperature 25°.

Although the precise nature of the inhibition of testosterone oxidation by estratrienes was not understood, it became of some interest to analyze the structural features required for binding to the enzyme surface.   These factors have in part been analyzed for β enzyme elsewhere (2).   The velocity of oxidation was determined in the absence of inhibitor, and in the presence of three inhibitor concentrations, in which the ratio of substrate (S) concentration to inhibitor (I) concentration was less than 1, equal to 1, and greater than 1.   The degree of inhibition obtained was plotted against S/I and the approximate values obtained for 1:1 ratio are given in Table IV.   Substrate and inhibitor concentrations were checked by enzymatic assay whenever possible.

It has been shown above that the requirements for an oxidizable substrate for β-hydroxysteroid dehydrogenase are the presence of a β oriented OH group at position 3, 16, or 17. In as far as ring D is concerned, a β-OH group at position 16 or 17 is only oxidizable provided the adjacent position is unsubstituted by a hydroxyl or ketone grouping. The estratrienes which cannot serve as substrates are nevertheless efficiently bound to the enzyme surface and this binding is dependent upon the presence of an intact phenolic 3-OH group, and to a much lesser extent on other substituents in ring D, although phenol itself is not an active inhibitor at much higher concentrations.

TABLE III

Michaelis Constants (K_s) for Diphosphopyridine Nucleotide with 3α-, 3β-, and 17β-Hydroxysteroids

Steroids	Enzyme	Position of oxidation	K_s, $\times 10^{-5}$ M	Concentration of steroid, $\times 10^{-5}$ M
Androstan-3α-ol-17-one..................	α	3α-OH	10.5	2.3
1,3,5-Estratrien-17β-ol..................	β	17β-OH	8	3.1
Testosterone...........................	"	"	5.5	0.65
5-Androsten-3β-ol-17-one................	"	3β-OH	1.1	1.39
Etiocholan-17β-ol-3-one................	"	17β-OH	0.33	13.5
Etiocholan-3β-ol-17-one................	"	3β-OH	0.26	1.6

3.0 ml. system containing 100 μmoles of sodium pyrophosphate buffer, pH 9.1, varying concentrations of DPN+, 2.7 γ of β enzyme (96,200 units per mg.) or 7.2 γ of α enzyme (3750 units per mg.), 0.08 ml. of CH_3OH, and concentrations of steroid as indicated. The reaction was initiated by addition of enzyme and optical density measurements were made in 1 cm. light path cells at 25° and at 340 mμ against a blank cell containing all ingredients except steroid.

As indicated in Table IV, the β enzyme action on both the 3-OH and 17-OH positions is strongly inhibited by most 1,3,5-estratrien-3-ol derivatives. Equimolar concentrations of substrate and inhibitor resulted in 70 to 90 per cent inhibition of the rate of oxidation of hydroxyl groups at both the 3β (androstan-3β-ol-17-one) and 17β (testosterone) positions. Absence of the hydroxyl group at the 17 position (1,3,5-estratrien-3-ol) did not quantitatively alter the inhibitory action of the estratrienes with intact phenolic A rings.

Three of the estratriene derivatives studied also possessed substrate properties with widely different characteristics. For example, estradiol-3,17β was oxidized at a maximal rate of 13.3 relative to testosterone (arbitrarily set equal to 100 at $S_0 = 6.0 \times 10^{-6}$ M). Estradiol-3,16β was also oxidized by β enzyme at a rate of 2.3 relative to testosterone. Both the

estradiol-3,17β and estradiol-3,16β exhibited strong affinity for the enzyme surface and thus could act as inhibitors of testosterone oxidation. The third estratriene utilized as a substrate, 1,3,5-estratrien-17β-ol, was oxidized at a maximal rate which was considerably greater ($V_0 = 135$) than the reference compound. Thus, inhibition could not be demonstrated with this compound, although experiments with its epimer served to elucidate the problem. 1,3,5-Estratrien-17α-ol was not oxidized by β enzyme
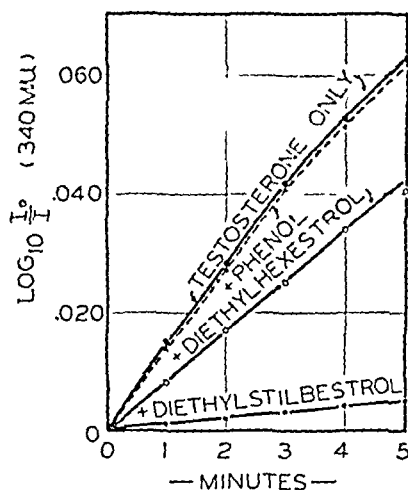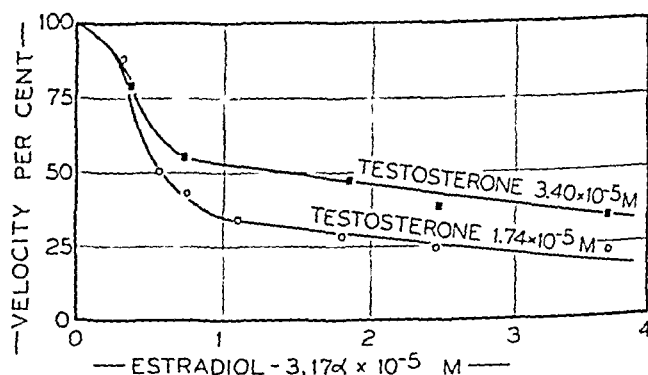
FIG. 7   FIG. 8

FIG. 7. Inhibition of the enzymatic oxidation of testosterone. Measurements were made of optical density at 340 mμ against time in a 3.0 ml. system containing 100 μmoles of pyrophosphate buffer, pH 9.1, 0.2 ml. of methanol, and 0.5 μmole of DPN$^+$. 14 units of β enzyme were used to initiate the reaction. Concentration of testosterone 1.16 $\times$ 10^{-5} M in each case. 1.8 $\times$ 10^{-4} M phenol; 3.1 $\times$ 10^{-5} M diethylhexestrol or 3.1 $\times$ 10^{-5} M diethylstilbestrol was added as indicated.

FIG. 8. Inhibitory effect of estradiol-3,17α on testosterone oxidation by β enzyme. The system contained, in 3.0 ml. volume, 100 μmoles of pyrophosphate buffer, pH 9.1, 0.5 μmole of DPN$^+$, 0.2 ml. of methanol, testosterone (concentration as indicated), and varying concentrations of estradiol-3,17α. Temperature 25°.

and, in sharp contrast to the phenolic estratrienes, showed only slight inhibitory action (less than 5 per cent).

It appears that the area on the estratriene nucleus most important for binding to β enzyme consists of the intact phenolic A ring. Removal of the 3-hydroxyl group results in almost complete loss of inhibitory action on both the 3β- and 17β-hydroxyl oxidations in the substrate-inhibitor concentration range studied. The introduction of a 17β-hydroxyl group into 1,3,5-estratriene lacking a 3-hydroxyl group results in a compound with excellent substrate properties.

A study of the effect of various substituents on the estratriene nucleus revealed the basic requirements for a molecule which either is an inhibitor

or is utilized as a substrate. By proper manipulation of substituents the estratriene nucleus can be made into a strong or weak inhibitor, a very active or only slightly active substrate, or a strong inhibitor with slight substrate activity.

TABLE IV

Inhibitory Effect of 1,3,5-Estratriene Derivatives on α and β Enzymes

Compound	Maximal velocity of oxidation by β enzyme*	Per cent inhibition of oxidation rate†		α enzyme, androsterone
		β enzyme		
		Testosterone	Epiandrosterone	
1,3,5-Estratriene-3,17α-diol........................	0	70	70	15
1,3,5-Estratriene-3,17β-diol........................	13.3	80‡	70‡	15
1,3,5-Estratriene-3,16α-diol........................	0	80	80	15
1,3,5-Estratriene-3,16β-diol........................	2.3	90‡	80‡	15
1,3,5-Estratriene-3,16α,17β-triol	0	90	90	15
1,3,5-Estratriene-3,16β,17β-triol.	0	80		
1,3,5-Estratriene-3,17β-diol-16-one	0	75		
1,3,5-Estratrien-3-ol............................	0	85	80	35
1,3,5-Estratrien-17α-ol...........................	0	5	5	5
1,3,5-Estratrien-17β-ol...........................	135			20

* None of these compounds is oxidized by α enzyme.

† The inhibitions are expressed as $(1 - V_i/V) \times 100$, where V and V_i are the velocities in the absence and presence of the inhibitor for equal concentrations of substrate and inhibitor. The system for measuring velocities contained, in a volume of 3.0 ml., 100 μmoles of pyrophosphate buffer, 10 γ of testosterone or 15 γ of epiandrosterone or 20 γ of androsterone, and 0 to 40 γ of inhibitor, 0.5 μmole of DPN⁻, 1 to 5 γ of enzyme, and 0.1 ml. of methanol. Final pH 9.1; temperature 25°. Measurements were made of optical density at 340 mμ against control cells containing all components except steroid. Velocities were obtained from the slopes of the zero order portions of the velocity-time curves.

‡ These figures are uncorrected for the oxidation rate of the estratriene derivative.

Table IV also shows the quantitative differences in the inhibitory effect of the eight estratrienes tested against α enzyme activity as measured by androsterone oxidation. Equimolar concentrations of substrate with most of the inhibitors reduce α enzyme activity by only about 15 per cent.

The absence of a hydroxyl group at the 17 position results in more than double the amount of inhibition (35 per cent). When the 3-hydroxyl is absent, the results depend upon the orientation of the 17-hydroxyl; with a 17α orientation there is only 5 per cent inhibition, whereas with 17β orientation the inhibition rises to 20 per cent.

Methods and Materials

Preparations of α and β enzymes of stated specific activities were obtained by methods described in an accompanying paper (1). DPN+ was purchased from the Pabst Laboratories, Milwaukee, Wisconsin, and was of 85 per cent purity on a weight basis. Triphosphopyridine nucleotide was obtained from the Sigma Chemical Company, St. Louis, Missouri. DPNH was prepared as its tris(hydroxymethyl)aminomethane salt according to Loewus, Westheimer, and Vennesland (11) and was a gift from Dr. Loewus. Steroids were generously donated by various investigators.[2] Criteria for identification and purity were established whenever possible by melting point, specific rotation, and enzymatic assay. Reaction rates were measured as described elsewhere (2).

DISCUSSION

Purified α- and β-hydroxysteroid dehydrogenases of bacterial origin serve as models for the study of the interaction of steroids with enzymatically active protein surfaces. The affinity of these enzymes for their steroidal substrates is high and relatively specific.

The physiological functions of these enzymes are not yet understood. Steroid dehydrogenases are widely distributed in nature, and, when their presence has not been directly demonstrated, it may be inferred from the types of steroid conversions observed. The interconversions of 3- and 17-ketosteroids and their respective β-hydroxysteroids have been demonstrated *in vivo*, in organ slices, and in homogenates (12) of various mammalian species. These interconversions are also carried out by such

diverse systems as yeasts (13), streptomyces (14), and by the adaptive enzymes of *Pseudomonas testosteroni*. An enzyme converting testosterone to 4-androstene-3,17-dione was demonstrated and partially fractionated from steer liver by Sweat *et al.* (15).

The direct application of spectrophotometric methods in the study of steroid dehydrogenases (2, 3) clearly demonstrated the direct participation of DPN+, the readily reversible nature of these reactions, and the conditions required for quantitative conversions. The properties of the bacterial β enzyme suggest that a single enzyme is concerned in the oxidation-reductions at C_3, C_{17}, and C_{16} of the steroid nucleus. Evidence favoring the unitary nature of this enzyme has been discussed elsewhere (2). Moreover, this enzyme is not only concerned in the oxidation of C-19 compounds, but also catalyzes the interconversion of estradiol-3,17β and estrone. The latter reaction has been reported *in vivo* and in various tissues (12). There is at present no contrary evidence that a single enzyme is responsible in mammalian tissues for all these conversions.

Evidence from metabolic studies has pointed to the existence of a 3α-hydroxysteroid dehydrogenase. Direct evidence for such an enzyme was first obtained in microorganism *P. testosteroni* (4). Halperin, Quastel, and Scholefield (16) demonstrated cholic acid breakdown by a soil *Nocardia* species and found that cell-free extracts of this microorganism catalyzed the DPN+-linked oxidation of the 3α-hydroxyl group. Tomkins and Isselbacher (17) obtained a fraction from rat liver which interconverts dihydrocortisone and tetrahydrocortisone by either oxidized triphosphopyridine nucleotide or DPN+ and demonstrated this reaction by direct spectrophotometry. Unpublished experiments by Mr. E. U. Thiessen in our laboratory have demonstrated that a DPN+-linked 3α-hydroxysteroid dehydrogenase interconverting androsterone and androstane-3,17-dione is present in rat uterus and vagina as well as in liver. The enzyme is in part soluble and in part associated with the microsomal fraction. Somewhat purified preparations of rat liver 3α-hydroxysteroid dehydrogenase catalyze the oxidation of pregnane-3α,17α,21-triol-11,20-dione as well as that of androsterone. It seems reasonable to assume that similar DPN+-requiring enzymes are involved in the reversible oxidations of all these 3α-hydroxysteroids.

Detailed consideration (2) of the binding of various related steroids to β enzyme has led to the conclusion that two reactive sites, which are concerned, respectively, with dehydrogenation reactions at positions 3 and 17, are present on the enzyme surface. The high degree of affinity, and the fact that minor structural modifications at some distance from the reactive sites (positions 3 and 17) profoundly influenced substrate binding, suggested a rather specific interaction between the enzyme and the substrate

extending over multiple points of attachment. Evidence was presented (2) that the rear surface of the steroid nucleus was concerned in this binding process, especially as it has been shown that in the dehydrogenations catalyzed by β enzyme at position 17 the 17α-hydrogen is directly transferred to and from the coenzyme (10).

SUMMARY

1. β-Hydroxysteroid dehydrogenase catalyzes the reversible DPN$^+$-linked oxidation of 3β-, 16β-, and 17β-hydroxysteroids.

2. α-Hydroxysteroid dehydrogenase catalyzes the reversible DPN$^+$-linked oxidation of 3α-hydroxysteroids of C-19, C-21, and C-24 series.

3. The rates of oxidation of various steroids by these enzymes have been measured.

4. The pH of the medium affects the equilibrium point and initial velocities of the reactions catalyzed by α and β enzymes. The equilibrium constant for the conversion of testosterone to 4-androstene-3,17-dione is 2.6×10^{-8} and that for the conversion of androsterone to androstane-3,17-dione is 5.8×10^{-9}.

5. The enzymes may be used for the specific enzymatic microassay of selected groupings on the steroid nucleus either singly or in combination. Examples of the measurement of 3α-hydroxyl groups and 3β- and 17β-hydroxyl groups are given and the use of these enzymes for enzymatic identifications is illustrated.

6. Michaelis constants of α and β enzymes for DPN$^+$ with various substrates have been determined.

7. β enzyme is strongly inhibited by estradiol-3,17β and certain related 1,3,5-estratrienes, as well as by diethylstilbestrol and diethylhexestrol. The structural requirements for β enzyme inhibitions are presented.

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THE EFFECT OF DIFFERENT DIETARY FATS ON LIVER FAT DEPOSITION*

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When weanling rats are fed a ration containing 9 per cent casein with sucrose as the carbohydrate and with an adequate supply of the known lipotropic factors, fatty infiltration of the liver occurs (1, 2). The addition of 0.36 per cent of DL-threonine to the diet causes a considerable decrease in liver fat deposition but does not reduce the liver fat content to as low a level as is found in rats fed an 18 per cent casein diet. Glycine fed at a level of 1.5 per cent of the diet also causes a reduction in the deposition of liver fat when it is fed either with or without supplemental threonine.

In a study of the effect of increased levels of dietary fat (3) it was found that the corn oil normally used in the diet could be increased from 5 to 20 per cent without causing any greater accumulation of liver fat. In fact, the liver fat content of rats fed the higher level of corn oil was slightly lower. The liver fat content of rats fed a low protein diet containing 20 per cent of butter fat was higher than that of rats fed a similar diet containing 20 per cent of corn oil. When threonine was added to the high fat diets, the difference between the levels of liver fat of the corn oil group and that of the comparable butter fat group was greater although the total amount of liver fat was reduced in each case.

Channon et al. (4, 5), in 1936, studied the effect of different dietary fats on liver fat deposition in rats fed low protein diets deficient in choline. Adult rats were used in their work and diets containing 5 per cent of casein and 40 per cent of fat were fed. They found the highest liver fat values for rats fed butter fat and the lowest for those fed olive oil; the values for those fed coconut oil were intermediate. From a comparison of the amounts of the different fatty acids in these fats they concluded that the increased infiltration of fat was proportional to the percentage of saturated C_{14} to C_{18} acids in the dietary fat.

Their work was complicated by both a very low protein intake and a

choline deficiency. They did not determine to what extent the accumulation of liver fat could be decreased by supplementation with choline or additional protein. The fatty acids were not fed free of the other components of the fats to implicate them directly in the increased liver fat deposition. The present work was undertaken to clarify these observations.

Methods

Male weanling rats of the Sprague-Dawley strain weighing 40 to 50 gm. were used in all of the experiments. They were divided according to weight into similar groups of six and housed in individual cages with raised screen bottoms. The animals were fed *ad libitum* and were weighed weekly during the 2 week experimental period.

The composition of the basal diet was as follows: sucrose 66.6, casein 9.0, DL-methionine 0.3, Salts IV (6) 4, choline chloride 0.15, and the fat specified in Table I 20 per cent. Vitamins were included to provide, in mg. per 100 gm. of ration, thiamine hydrochloride 0.5, riboflavin 0.5, niacin 1.0, calcium pantothenate 2.0, pyrixodine 0.25, biotin 0.01, folic acid 0.02, vitamin B_{12} 0.02, and inositol 10.0. 2 drops of halibut liver oil fortified with vitamins E and K (1) were administered orally each week. Alterations in the amount of sucrose compensated for the alterations in the basal diet.

At the end of the 2 week experimental period the animals were sacrificed for the determination of liver fat. The animals were stunned by a blow on the head and decapitated. The livers were removed and stored at $-4°$ until the fat determinations could be carried out. Liver fat was determined by extraction of the dried and ground liver with ether (7).

All of the fats used were commercial products. Both the butter and the margarine were melted and the fat layers were decanted and strained through cheese-cloth.

The unsaponifiable fraction of butter was prepared by heating 450 gm. of butter with 3 liters of 1 N alcoholic potassium hydroxide under reflux for 2 hours. An equal volume of water was added and the resulting solution was extracted twice with diethyl ether. The ether layer was washed three times with water and heated to dryness at 80°. The yield was 2.8 gm. or 0.62 per cent of the butter fat. This was fed as 0.14 per cent of the diet.

The water layer and washings from the previous procedure were acidified with concentrated hydrochloric acid and the fatty acid layer was separated. The water and alcohol were removed by heating at 80°. The fatty acids of corn oil were prepared by the same procedure.

For the esterification of fatty acids, 180 gm. of the fatty acids were

heated with 38 gm. of glycerol, and the progress of the reaction was followed by observing the amount of water which was distilled. Nitrogen gas was bubbled through the mixture during the heating period to prevent oxidation. A trace of zinc (8) was added as a catalyst. The product was taken up in ether and washed twice with water. Ether and water were removed under a vacuum. The resulting product was fed at a level of 20 per cent in the diet.

The liquid and solid fractions of the fatty acids of butter were prepared by the lead salt separation as described by Hilditch (9). The fatty acid fractions were esterified by the procedure given above.

Results

The results of each experiment are presented separately in order that each group may be compared with the control in the same experiment. Although the liver fat values are usually consistent within experiments, considerable variation is encountered among similar groups in different experiments.

In Experiment 1, the liver fat values for the groups fed corn oil and margarine were considerably lower than those for groups fed butter fat or lard. Although threonine reduced the level of liver fat in each case, this difference occurred whether threonine was added or not. The liver fat value for Group 2 which received corn oil and threonine was higher than those of similar groups in the other experiments. The liver fat values for the groups that received 10 per cent of corn oil and 10 per cent of butter fat were intermediate between those for the groups receiving corn oil and the groups receiving butter fat. The levels of liver fat of Groups 11 and 12 which received the 18 per cent casein diet were very low, and there was no difference between the liver fat levels of the groups receiving the different fats.

A supplement of 1.5 per cent of glycine caused a reduction in the level of liver fat in the animals that received threonine and in those that did not (Experiment 2). However, the difference between the liver fat content of those receiving corn oil and those receiving butter fat was reduced only when both threonine and glycine were included in the diet.

In Experiment 3, when the unsaponifiable fraction of butter was fed with corn oil, the level of liver fat was not increased. In an experiment not presented in Table I, 75 mg. per cent of cholesterol or 2 per cent of sodium butyrate was found to have no effect on liver fat deposition when fed with 20 per cent of corn oil. Also, the food consumption of the groups receiving corn oil and butter fat was found to be identical, although there was a significant difference in the level of liver fat.

The fatty acids of butter and of corn oil were esterified with glycerol and

TABLE I

Effect of Dietary Fat on Liver Fat Deposition

Experiment No.	Group No.	Fat, 20%	DL-Threonine	Changes in basal diet	Rate of gain*	Liver fat*	
			per cent		*gm. per wk.*	Dry weight *per cent*	Wet weight *per cent*
1	1	Corn oil			15.1 ± 1.2	22.4 ± 1.7	6.7 ± 0.6
	2	" "	0.36		21.0 ± 1.4	19.4 ± 1.8	5.7 ± 0.6
	3	Butter fat			12.7 ± 0.9	32.6 ± 3.1	10.6 ± 1.3
	4	" "	0.36		18.4 ± 0.8	24.2 ± 1.5	7.4 ± 0.6
	5	Margarine			13.5 ± 0.8	22.7 ± 1.4	6.8 ± 0.6
	6	"	0.36		19.7 ± 1.6	12.9 ± 0.7	3.5 ± 0.2
	7	Lard			13.8 ± 0.6	26.2 ± 1.3	8.2 ± 0.6
	8	"	0.36		20.0 ± 0.7	20.3 ± 1.4	5.9 ± 0.5
	9	Butter fat, 10% Corn oil, 10%			12.8 ± 0.8	28.0 ± 1.8	8.8 ± 0.7
	10	Butter fat, 10% Corn oil, 10%	0.36		19.0 ± 1.2	19.8 ± 1.4	5.9 ± 0.5
2	11	Butter fat		18% casein†	37.6 ± 1.3	12.6 ± 0.3	3.5 ± 0.2
	12	Corn oil		18% "	34.8 ± 1.2	14.8 ± 1.0	4.1 ± 0.3
	13	Butter fat	0.36		21.4 ± 0.6	17.5 ± 0.7	5.1 ± 0.2
	14	Corn oil	0.36		20.0 ± 0.8	22.6 ± 1.7	6.9 ± 0.6
	15	Butter fat	0.36	1.5% glycine	21.8 ± 1.3	16.3 ± 0.9	4.6 ± 0.3
	16	Corn oil	0.36	1.5% "	24.9 ± 0.9	17.2 ± 2.1	5.1 ± 0.8
	17	Butter fat		1.5% "	16.3 ± 1.3	19.1 ± 0.5	5.5 ± 0.2
	18	Corn oil		1.5% "	16.9 ± 1.0	26.6 ± 2.2	8.2 ± 0.8
3	19	Corn oil	0.36		21.1 ± 0.4	15.6 ± 0.8	4.8 ± 0.3
	20	" "	0.36		20.3 ± 0.5	13.2 ± 1.0	4.0 ± 0.3
	21	Butter fat	0.36	Unsaponifiable of butter	20.9 ± 0.7	20.9 ± 1.7	6.9 ± 0.7

	No.	Diet	Casein				
4	22	Corn oil		0.36	24.1 ± 1.4	16.6 ± 1.5	5.0 ± 0.5
	23	Butter fat		0.36	24.4 ± 1.2	24.3 ± 1.0	7.3 ± 0.3
	24	Fatty acids of butter‡		0.36	19.7 ± 0.8	22.8 ± 3.2	7.1 ± 1.2
	25	" " corn oil‡	7% casein†	0.36	17.0 ± 0.5	15.5 ± 0.6	4.3 ± 0.2
	26	Corn oil	7% "	0.36	14.4 ± 1.1	17.2 ± 1.1	4.9 ± 0.3
	27	Butter fat	7% "	0.36	10.1 ± 0.6	29.5 ± 3.2	0.4 ± 1.3
	28	Corn oil	7% " ; no choline	0.36	12.8 ± 0.7	38.2 ± 2.9	13.4 ± 1.5
	29	Butter fat	7% " "	0.36	18.4 ± 1.2	55.1 ± 1.8	23.0 ± 1.2
5	30	Corn oil	7% " "		9.6 ± 0.4	34.5 ± 1.8	11.7 ± 0.8
	31	Butter fat	7% " "		12.6 ± 1.1	58.2 ± 2.2	25.0 ± 1.7
	32	Fatty acids of butter ‡	7% " "		11.9 ± 0.8	59.0 ± 2.5	25.7 ± 2.0
	33	Liquid " " ‡	7% " "		14.1 ± 0.6	44.6 ± 2.8	16.3 ± 1.5
	34§	Solid " " ‡	7% " "		9.7 ± 0.3	66.8 ± 0.9	31.4 ± 0.5
6	35	Corn oil	7% " "	0.36	13.1 ± 1.3	34.2 ± 1.7	11.5 ± 0.7
	36	Butter fat	7% " "	0.36	14.2 ± 0.8	59.0 ± 1.5	26.1 ± 1.1
	37	Oleic acid‡	7% " "	0.36	10.2 ± 0.6	28.6 ± 2.2	9.2 ± 0.8
	38	" + stearic acids‡ (1:1)	7% " "	0.36	11.8 ± 0.5	52.7 ± 1.8	21.1 ± 0.9

* The values represent the mean ± the standard error of the mean for six animals.
† This is the total amount of casein in the diet.
‡ The fatty acids were esterified with glycerol and the product was fed as 20 per cent of the diet.
§ One animal died and another lost weight during the 2nd week. The results presented are for only four animals.

fed to rats as 20 per cent of the diet in Experiment 4. The levels of liver fat of rats fed these preparations were the same as those of the groups receiving the fats from which they were prepared.

When the level of casein was decreased (Groups 26 and 27) from 9 to 7 per cent, the difference between the liver fat content of the groups receiving corn oil and of those receiving butter fat was increased. An even greater difference was obtained when the lower level of casein was fed and choline was omitted from the diets (Groups 28 and 29). There was also a marked increase in the level of liver fat in both groups when choline was omitted.

In the remaining experiments, the 7 per cent casein diet without choline was used to insure larger and more consistent differences. The level of liver fat of the groups fed the glycerides of the fatty acids from butter in Experiment 5 was as high as that of the group which received butter fat. The solid or long chain saturated fatty acids of butter, fed as the glycerides, caused a higher level of liver fat than did butter fat. The level of liver fat of the rats receiving the liquid fatty acid glycerides was below that of the group receiving butter fat, but was still above the level of those receiving corn oil.

Stearic acid was chosen as a member of the solid fatty acid group to be tested for its effect on liver fat deposition. A mixture of stearic and oleic acids (1 : 1) was used to give a product with a lower melting point and this was compared with the glycerides prepared from oleic acid alone. The level of liver fat of the group that received stearic acid was nearly as high as that of the group fed butter fat, while the group which received oleic acid alone showed a level of liver fat below that of the corn oil group.

DISCUSSION

Previous work has shown that the level of corn oil fed to rats receiving the 9 per cent casein diet containing choline did not affect the severity of the fatty infiltration of the liver even when the level was varied from none to 20 per cent. Butter fat or lard, when fed as 20 per cent of the diet, caused a marked increase in the level of liver fat. When a low protein choline-deficient diet was fed, the difference between the liver fat content of the group receiving butter fat and that of the group receiving corn oil was greater than that observed when diets containing choline were used. Although differences (1, 10) have been observed between the fatty livers which result from a protein deficiency and those caused by a choline deficiency, in both cases the effect of different dietary fats on the level of liver fat is similar.

Butter fat does not affect the level of liver fat when the diet contains adequate levels of protein and choline. When either protein or choline is

inadequate, butter fat added to the diet increases the severity of the fatty infiltrations of the liver which result under these dietary conditions. In general, this effect of butter fat was more pronounced when the diet was made more deficient in lipotropic factors. The choline-deficient diet which was used in this work produced the highest level of liver fat. Also, the greatest difference between the liver fat content of the group receiving butter fat and of that receiving corn oil was found in the experiment in which this type of diet was used. This difference in liver fat content between the butter fat and corn oil groups was also increased when the level of protein in the diets containing choline was reduced from 9 to 7 per cent. This was accompanied by an increase in the level of liver fat in the group fed corn oil. This generalization did not hold for the groups receiving the 9 per cent casein sucrose diet containing choline and supplemented with threonine or glycine, for these compounds reduced the level of liver fat but did not reduce the difference between the liver fat levels in the groups fed butter fat and those fed corn oil.

Isolation and fractionation of the fatty acids of butter by the lead salt procedure clearly demonstrated that the fatty acids and in particular the long chain saturated fatty acids of butter cause an increase in liver fat deposition when fed at rather high levels in diets deficient in protein and choline. Corn oil contains a very small amount (about 13 per cent) of the long chain saturated acids and therefore should have little influence on the accumulation of liver fat as was found in this work. These results support the suggestion of Channon *et al.* (5) that the level of C_{14} to C_{18} saturated fatty acids affects the amount of liver fat which will accumulate.

Stearic acid was found to be active in increasing liver fat deposition but other saturated fatty acids have not been tested. The liquid fatty acids of butter caused a higher level of liver fat than did corn oil. This may be explained by the amounts of myristic and palmitic acids which remained in this fraction or may indicate an effect from the shorter chain saturated acids.

The marked increase in liver fat deposition caused by the long chain saturated fatty acids indicates that these acids are metabolized by the liver in a manner different from that of the unsaturated acids which do not affect the accumulation of fat in the liver. The present knowledge of the causes of fatty infiltration of the liver is not complete enough to permit any conclusion as to what the difference in the metabolism of the two groups of fatty acids may be.

This effect of fats containing large amounts of long chain saturated fatty acids on liver fat deposition would be of importance only when relatively large amounts of fat were fed to animals receiving diets deficient in either choline or protein. For example, the amount of fat accumulating in the

livers of two groups of rats receiving high fat diets containing the same *suboptimal* level of choline would be distinctly different if the dietary fats for the two groups differed appreciably in their content of saturated fatty acids.   On the other hand when an adequate diet is consumed, as is usually the case under practical conditions when the intake of animal fats is high, the type of fat would be without effect on the deposition of liver fat.

SUMMARY

When different fats were fed at a level of 20 per cent in a low protein diet (9 per cent casein), the level of liver fat in the rats was high when butter or lard was fed and was low when corn oil or margarine was fed. This effect was increased when the level of protein in the diet was decreased and when choline was omitted from the diet.

The fatty acids of butter, which were isolated and fed as glycerides, caused liver fat to accumulate to the same extent as did butter fat.   The solid fatty acid fraction of butter caused a much greater accumulation of liver fat than did the liquid fatty acid fraction, and stearic acid caused a much greater accumulation of liver fat than did oleic acid.   The unsaponifiable material of butter was without effect.

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ON THE MECHANISM OF DEHYDROGENATION OF FATTY ACYL DERIVATIVES OF COENZYME A

I. THE GENERAL FATTY ACYL COENZYME A DEHYDROGENASE

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The first step in the degradation of fatty acyl CoA[1] derivatives is a dehydrogenation leading to the corresponding α,β-unsaturated derivatives (1–3).[2] In the early exploratory work carried out in this laboratory, Mii and Green (4) recognized that there were two separate enzymes capable of catalyzing this dehydrogenation. One, characterized by a green color (G), has already been described in detail by Green *et al.* (2). This enzyme is a cuproflavoprotein as shown by Mahler (5), and its range of catalytic activity is limited to fatty acyl CoA derivatives from C_4 to C_8. The present communication deals with a dehydrogenating enzyme of broader specificity, a flavoprotein of yellow color (Y), which acts on acyl CoA derivatives from C_4 to C_{16}. An additional yellow flavoprotein (Y') was recently discovered, which is specific for fatty acyl CoA derivatives of long carbon chain, but otherwise is similar to Y (6). This enzyme will be designated palmityl CoA dehydrogenase[3] and will be dealt with in a subsequent publication.

Like other flavoproteins, Y is readily reduced by its substrates, but has the unique property among flavoproteins of being highly specific for the oxidant. Y, when reduced by substrate to YH_2, cannot be reoxidized by conventional electron acceptors such as oxygen, ferricyanide, indophenol,[1] or cytochrome *c*. A variety of quinones will reoxidize YH_2 at a relatively slow rate, whereas N-methylphenazine does so at a more rapid rate. The interaction of YH_2 with all these electron acceptors can, however, be

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[1] The following abbreviations are used: coenzyme A, CoA; reduced CoA, CoASH; flavin adenine dinucleotide, FAD; 2,6-dichlorophenolindophenol, indophenol; tris-(hydroxymethyl)aminomethane, Tris; diphosphopyridine nucleotide, DPN. For G, Y, Y', and ETF see the introduction.

[2] The possibility that the β,γ-unsaturated derivatives are the primary products of dehydrogenation is not excluded (2).

[3] The name palmityl CoA dehydrogenase is preferred for purposes of differentiation, although the enzyme has a somewhat higher affinity for lauryl CoA.

catalyzed efficiently by yet another flavoprotein, which we will call the electron-transferring flavoprotein (ETF) (7). ETF serves as the specific oxidizing agent for YH_2. The reduced form of ETF (ETFH₂) is then in turn reoxidized by the electron acceptors mentioned above. Dehydrogenation of the substrate is the function of Y and interaction with electron acceptors is the function of ETFH₂ in this two-enzyme system.[4]

In the present communication the purification and properties of Y from pig liver will be described, while an accompanying paper will deal with the purification and properties of ETF (8).

<div align="center">EXPERIMENTAL</div>

<div align="center">*Methods*</div>

Substrates—Acyl derivatives of CoA were prepared enzymatically by the use of the appropriate activating enzymes (9, 10)[5] or chemically by a modification of the anhydride method described for succinyl CoA by Simon and Shemin (11).[6]

Spectrophotometry—Spectra were taken with a Beckman model DU spectrophotometer equipped with a micro attachment (12) and a temperature-controlled cell compartment which was kept at 30° during rate measurements and cooled with ice water when spectra were taken.

Protein—Protein was determined by the biuret reaction (13). When Tris buffer was present, the protein was first precipitated with trichloroacetic acid, centrifuged in a micro centrifuge tube, and redissolved in biuret reagent. The biuret values were standardized against a determination of dry weight.

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[4] Although this type of relationship between flavoproteins has been more thoroughly studied with Y and ETF, a reinvestigation of G showed that ETF is also an obligatory electron carrier in the catalytic turnover of G. The same holds true for Y′, the yellow palmityl CoA dehydrogenase referred to above.

[5] We are indebted to Dr. P. G. Stansly for a gift of enzymatically prepared palmityl CoA.

[6] It should be pointed out here that preparations of CoA derivatives obtained by the two methods differ in so far as they contain impurities of a different nature. Most available CoA preparations contain glutathione, which is readily converted to the thioester if one uses the synthetic methods for acyl CoA preparation, whereas the activating enzymes are specific for CoA and will not acylate glutathione. In tissues there exist thioesterases of varied specificity. Liberation of free sulfhydryl will therefore be different with the two types of CoA derivatives. It was found in this work that specific glutathione thioesterases are abundant in liver extracts. They are sufficiently active even in preparations of Y of high purity to make an assay with electron acceptors such as dyes or cytochrome c impossible if the synthetically prepared CoA derivatives are used. On the other hand, glutathione thioesterases can be used to free CoA derivatives from contaminating acyl derivatives of glutathione.

Results

Assay of Enzyme Activity—As pointed out in the introduction, two flavoproteins, Y and ETF, are involved in the catalysis of the over-all oxidation of acyl CoA derivatives by electron acceptors. The rate of reduction of an acceptor, *e.g.* indophenol or cytochrome *c*, will therefore only measure the activity of the particular enzyme which is present in limiting amounts. Assays with a number of acceptors can be used for the determination of Y, provided an excess of ETF is present. Conversely, ETF can be determined in the same system if an excess of Y is added. An additional complication arises from the fact that three primary dehydrogenases with overlapping specificity, G, Y, and Y', may be present. The differentiation between these dehydrogenases will be discussed below.

We have called the above type of assay the "catalytic assay." In applying the "catalytic assay," the possibility of non-enzymatic reduction of electron acceptors by CoA in its sulfhydryl form has to be ruled out. If the fraction to be tested contains enzymes which catalyze the deacylation of acyl CoA derivatives to CoASH (14), then the CoASH so generated is capable of reacting spontaneously with certain acceptors like cytochrome *c* or indophenol.[7] The systems used for the "catalytic assay" have been described previously (2, 4). In the present work the assays with all acceptors are carried out in 0.02 M phosphate buffer of pH 7.0. The reaction is started by adding 0.05 μmole of butyryl CoA or 0.02 μmole of octanoyl, lauryl, or palmityl CoA. The total volume of the reaction mixture is 0.5 ml. The oxidation is not inhibited by phosphate ions. 1 unit of activity with indophenol is defined as that amount of enzyme which produces a change in optical density of -1.00 at 600 mμ in 1 minute in 0.5 ml. of reaction mixture. Specific activity equals units per mg. of protein.

Since there may be as many as three primary dehydrogenases present in the material under investigation, a single assay, carried out as described, may not be a valid measure of Y. At present an exact knowledge of the specificity pattern of the dehydrogenases is only available for pig liver. The specificity of the pig liver enzymes has been recently reported (6) and will be dealt with in the appropriate section. In order to make the "catalytic assay" a valid measure of Y, the following criteria have been applied. G is most active with butyryl CoA as substrate and is inert toward palmityl CoA as substrate and as inhibitor. Y has its peak of activity with the substrates of 8 to 12 carbons in the fatty acid residue and oxidizes butyryl CoA and palmityl CoA at about one-fourth to one-fifth this rate. It is characteristic of Y that trace amounts of palmityl CoA or

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[7] The considerations of foot-note 6 should also be taken into account here.

its product of enzymatic dehydrogenation will inhibit butyryl CoA oxidation completely. Y' will not oxidize butyryl CoA at a significant rate under any conditions. This inertness toward butyryl CoA and the fact that Y' oxidizes palmityl CoA at 90 per cent of the rate shown with octanoyl CoA readily differentiate Y' from Y. Therefore, if all three dehydrogenases are present and a quantitative estimate is desired, assays with (a)

TABLE I

Purification of Y from Pig Liver

Fractionation step	Protein	Specific activity*		Units		Recovery	
		C_4†	C_{16}†	C_4	C_{16}	C_4	C_{16}
	mg.					*per cent*	*per cent*
Extraction of 300 gm. acetone powder of pig liver mitochondria	66,000	0.7	0.3	45,000	19,800	100	100
Ammonium sulfate	8,000	1.4	0.86	11,000	6,900	24.5‡	35‡
Heating at 57°	3,560	2.2	1.5	7,840	5,350	17.5	27
Zn⁺⁺-ethanol	290	5.6	4.0	1,630	1,160	3.6	5.8‡
Ammonium sulfate at pH 8.1	84	6.6	6.3	555§	530	1.2	2.7
Zone electrophoresis	18	15.7	14.3	280§	260	0.6§	1.3‡

* Specific activity as defined in the text with indophenol as acceptor and in the presence of an excess of ETF.

† Assayed with butyryl CoA or palmityl CoA as substrate, respectively.

‡ It should be pointed out that 100 per cent for the extract includes Y and G when butyryl CoA is the substrate and Y and Y' when palmityl CoA is the substrate. Part of Y' and G is lost in the first ammonium sulfate step, and the remaining Y' is eliminated with Zn⁺⁺ in the fourth step. The total recovery of Y + Y' after zone electrophoresis when both enzymes are prepared from the same batch of acetone powder is 3.8 per cent. G is only separated on zone electrophoresis.

§ In addition about 270 units of G were obtained on zone electrophoresis of the ammonium sulfate fraction (55 to 60 per cent saturation) containing 555 units. The total recovery of C_4 units including G + Y is therefore 1.2 per cent.

butyryl CoA, (b) butyryl CoA in the presence of a trace of palmityl CoA, (c) palmityl CoA, and (d) octanoyl CoA are necessary.

As shown by the different behavior of the three enzymes in the fractionation procedure outlined below, specific preparative steps may also serve to single out, identify, or exclude individual enzymes.

Purification—The purification procedure used for Y is outlined in Table I. This procedure permits the isolation of Y, Y', and G from one and the same batch of pig liver mitochondrial acetone powder. Because of the close association of the three dehydrogenases, many fractions of high activity have to be eliminated, and the purification is carried on only with

fractions which show satisfactory separation. The low yield of the procedure is referable to this necessity. All manipulations are carried out at 0–4° unless otherwise specified. Solid ammonium sulfate is used in all ammonium sulfate fractionations. Saturation is calculated for ammonium sulfate solutions at 0°, 69.7 gm. per 100 ml. of water corresponding to 100 per cent saturation. The pH is adjusted only where so indicated.

300 gm. of acetone powder of pig liver mitochondria are extracted with 3 liters of 0.02 M phosphate of pH 7.8 for 30 minutes. After centrifugation at 2400 r.p.m. for 40 minutes in a 4 liter centrifuge (International Equipment Company) about 1.8 liters of a reddish brown supernatant fluid can be decanted, which is not always completely clear. This extract, which contains 20 to 25 mg. of protein per ml., is fractionated with ammonium sulfate without adjustment of pH into fractions of 40, 55, 65, and 75 per cent saturation. The fractions are taken up in small volumes of 0.02 M KHCO$_3$. The fraction obtained at 65 to 75 per cent saturation is used for the preparation of Y′, as will be described in a later communication. The 55 to 65 per cent fraction (about 100 ml. containing 60 mg. of protein per ml.) is dialyzed overnight against 0.02 M Tris acetate of pH 7.2. The dialyzed material (25 to 30 mg. of protein per ml.) is heated in a water bath at about 70° until it reaches a temperature of 57°. It is held at this temperature for 2 minutes and then immediately cooled in an ice bath. This procedure takes about 7 minutes from the time the mixture is brought above ice bath temperature until it again reaches ice bath temperature. The denatured protein is removed by centrifugation. The supernatant solution has a protein concentration of 15 to 20 mg. per ml. and a pH of about 6.9. A 0.05 M solution of zinc lactate of pH 5.5 is then slowly added with stirring until a concentration of 0.001 M is obtained. The pH of the mixture drops to 6.6. The precipitate which is formed is removed by centrifugation. This precipitate contains the remaining Y′ and may be used as a source of this enzyme. The supernatant solution of the zinc precipitate is then fractionated with cold absolute ethanol, and the temperature is kept slightly above the freezing point of the mixture. Fractions are separated by centrifugation at ethanol concentrations of 9, 12, 18, and 23 per cent, respectively. The precipitated zinc-protein complexes are dissolved in 0.4 M citrate of pH 7.2 and dialyzed immediately against a solution, 0.02 M with respect to Tris acetate, 0.001 M with respect to Versene, and of pH 7.2. After dialysis, the fraction which was obtained at 18 to 23 per cent ethanol is adjusted to a protein concentration of about 15 mg. per ml. with 0.2 M Tris acetate of pH 8.1, which usually requires the addition of an equal volume of this buffer. The protein solution is then fractionated at this pH with ammonium sulfate into four fractions between 50 and 65 per cent saturation. The fractions are dissolved in the smallest amounts possible of 0.02 M Tris acetate of pH 7.2.

The fraction obtained between 55 and 60 per cent saturation is then dialyzed with internal stirring for 3 hours against 0.2 M Tris acetate of pH 8.1. The dialyzed solution is subjected to zone electrophoresis on starch in the apparatus described by Flodin and Porath (15). The starch column is washed with the same buffer and placed in a cold room. 1 to 3 ml. of the dialyzed enzyme solution is then layered on top of the column. By slowly draining liquid from the column, the narrow yellow band of enzyme is moved down into the column a few cm., buffer being replaced on the top as necessary. The whole assembly is placed in a stirred water bath of about 4°. The negative electrode is attached to the top of the column. At a voltage of 600 volts and a current of 40 to 50 ma. satisfactory separation is achieved in 18 hours. G migrates about 15 cm. down the column, whereas Y moves only a few cm. ETF migrates in the opposite direction under these conditions and is found in the liquid above the column. The pattern of mobilities corresponds to that observed in the Tiselius apparatus, if the electroosmotic flow is taken into consideration, which is relatively high under these conditions. The bands are eluted from the column with the same buffer that was used during the separation and are collected in fractions of 2 to 3 ml. Each of the main fractions of the separated enzymes is thus obtained in a volume of about 6 to 10 ml. The enzyme protein is precipitated with ammonium sulfate and taken up in a small volume of 0.02 M $KHCO_3$.

Properties. (a) *Purity*—After zone electrophoresis on starch, Y is free of Y′, G, ETF, other enzymes of the fatty acid cycle, and glutathione thioesterases, enzymes which may cause considerable interference in assays of Y.[6] A preparation of Y which was examined during boundary electrophoresis in 0.2 M Tris acetate of pH 8.2 showed only one symmetrical boundary under these conditions (Fig. 1) which migrated toward the anode. The same preparation was studied in the analytical ultracentrifuge (Fig. 2). Only one peak was observed under these conditions (see the legend to Fig. 2), which was symmetrical and sedimented with the yellow color. The sedimentation coefficient, computed on the basis of this experiment and corrected[8] to water at 20° was $s_{20,w} = 7.66 \pm 0.06$ S. This value indicates a minimal molecular weight of 140,000 and a probable molecular weight close to 200,000.[9] The spectral properties, which will be discussed below, also indicate that the preparation of Y is of high purity.

[8] The formula $s_{20,w} = s_{\text{experimental}} \times \eta_{\text{experimental}}/\eta_{20,w}$ was applied for this correction.

[9] Computed for a spherical molecule whose hydrodynamic volume can be represented by Mv/N, where M is the molecular weight in gm. $\times$ mole^{-1}, N is Avogadro's number, and v, the partial specific volume, has been taken to be 0.74 ml. $\times$ gm.$^{-1}$. The probable molecular weight is calculated by assuming a frictional ratio, f/f_0, of 1.25.

(b) *Absorption Spectrum*—The absorption spectrum of Y is shown in Fig. 3. The typical flavoprotein peaks are of very high symmetry in all preparations which have been obtained by electrophoretic separation. The ratio of the absorbance at the maxima or minimum, respectively, at 275, 315, 370, and 447mμ was 12.9:0.43:0.74:1.00 in the preparation which
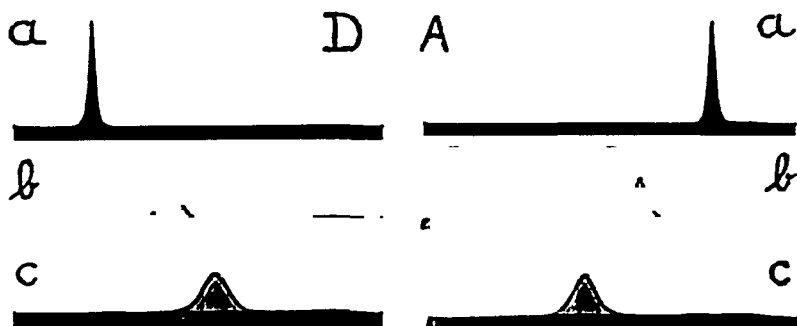
FIG. 1. Schlieren diagram of Y from pig liver obtained in a Spinco model H electrophoresis unit. 10 mg. of protein were used in 0.2 M Tris acetate, pH 8.2, in the micro cell of the apparatus. *A*, ascending limb (anode); *D*, descending limb. Pictures taken at 0 (*a*), 110 (*b*), and 186 (*c*) minutes.
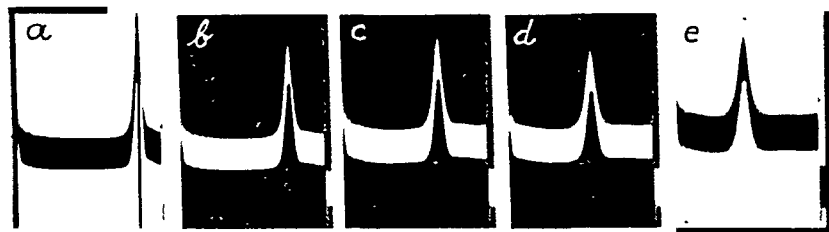
FIG. 2. Schlieren diagram of Y from pig liver in a Spinco model E analytical ultracentrifuge (mean temperature, 5.7°; rotor speed, 59,780 r.p.m.; buffer, 0.05 M potassium phosphate, pH 7.0; initial protein concentration, 10.3 mg. per ml.). Pictures taken at 30 (*a*), 46 (*b*), 62 (*c*), 78 (*d*), and 94 (*e*) minutes.

was studied in the ultracentrifuge and in the Tiselius apparatus as described above. However, preparations obtained by somewhat different procedures likewise exhibited symmetrical peaks but a lower value for the $E_{275}:E_{447}$ ratio. A ratio as low as 6.5 was observed in one case. It is very unlikely that the preparation which had a value of 12.9 for $E_{275}:E_{447}$ and which was studied during electrophoresis and ultracentrifugation was only about 50 per cent pure. If a loss of flavin during preparation was responsible for the high ratio obtained, one would expect a lower specific activity for this preparation and stimulation by added flavin. This was not the

case. Preparations of Y of a low $E_{275}:E_{447}$ ratio were not studied in the ultracentrifuge. It can therefore only be concluded at the moment that the preparative procedure used determines to a certain extent the spectral characteristics of Y and whatever properties are responsible for these characteristics.

When a fatty acyl CoA is added to Y, the prosthetic flavin is bleached. The extent of bleaching is determined by the chain length of the fatty acid residue of the substrate (see Table II). There is a strong increase in absorption between 500 and 650 mμ when Y is reduced with substrate. On
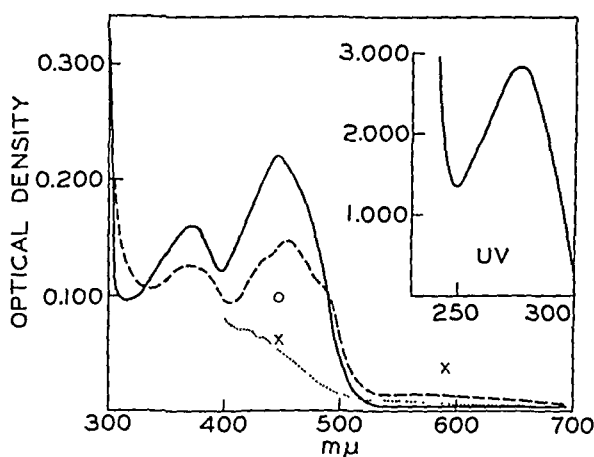
FIG. 3. Absorption spectrum of Y from pig liver. 295 γ of protein (specific activity 14.3) were dissolved in 0.14 ml. of 0.036 M phosphate, pH 7.5. Solid line, oxidized form; dash line, reduced with 0.05 μmole of palmityl CoA; dotted line, dithionite added after palmityl CoA. $\times$, optical density values at corresponding wave-lengths obtained with 0.02 μmole of octanoyl CoA. The rise of absorption at 590 mμ with this substrate, as discussed in the text, is clearly visible. O, extent of bleaching with 0.02 μmole of butyryl CoA at 447 mμ (not corrected for a small contribution of palmityl CoA between 300 and 320 mμ).

bleaching with dithionite, this band does not appear.[10] It can, however, be produced when an α,β-unsaturated fatty acyl CoA is added to the dithionite-reduced enzyme. Once the band has been produced by reduction of Y with substrate, the addition of dithionite leads only to a small and slow decrease in intensity of the band. In a similar way, dithionite leads to little further change of the residual absorption at 447 mμ when Y has been reduced by substrate (C_8 to C_{12}). In the absence of substrate, however, dithionite will bleach Y more extensively at 447 mμ than does substrate. Addition of an α,β-unsaturated fatty acyl CoA to the dithionite-bleached enzyme will then again restore the absorption at 447 mμ to the

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[10] A transient appearance of the band within the first few seconds after addition of dithionite was observed.

level which is reached by reduction with substrate alone. It is evident from these observations that the same interaction of YH_2 with the oxidized substrate which causes the appearance of the band between 500 and 650 mμ influences also the light absorption in the spectral region of the flavin peaks. The extent of bleaching of the prosthetic flavin of Y by substrate has therefore to be evaluated with caution.

(c) *Flavin Component*—A solution of Y ($E_{275}:E_{447}$ = 12.9) was acidified with trichloroacetic acid, cleared by centrifugation, and neutralized. The absorption at 450 mμ was measured before and after reduction with dithionite. The evaluation of this measurement for the flavin content of Y was based on the molar extinction coefficient for FAD (11.3 $\times$ 10^6 sq. cm. $\times$ mole^{-1} for the total absorption of FAD at 450 mμ (16)). From these measurements a riboflavin content of 0.22 $\pm$ 0.02 per cent was calculated for Y, corresponding to a minimal molecular weight of 160,000 to 190,000. As the sedimentation coefficient indicates a probable molecular weight of this magnitude, it may be tentatively concluded that Y from pig liver has 1 flavin per molecule. The prosthetic flavin is inseparable from chromatographically pure FAD[11] when chromatographed in two solvent systems (K_2HPO_4-amyl alcohol (17), R_F = 0.41; tertiary butanol-water[11] (60:40), R_F = 0.18). The flavin released from Y was tested with D-amino acid apooxidase from pig kidney (18) at a variety of concentrations. From the conventional double reciprocal plot (19) an apparent dissociation constant of 2.0 $\times$ 10^{-7} M ($\pm$7 per cent) was derived, compared to a value of 1.9 $\times$ 10^{-7} M ($\pm$10 per cent) for pure FAD under the same conditions. Further identification of the prosthetic flavin was not carried out. The available evidence is, however, consistent with the view that FAD is the prosthetic group of Y.

(d) *Metal Content*—When preparations of Y were dialyzed exhaustively against 0.02 M Tris acetate of pH 7.2 with or without added 0.001 M Versene, wet ashed with nitric acid, and then tested with o-phenanthroline and a reducing agent, the ratio atoms of iron to molecules of flavin was about 1:6. Negligible amounts of copper were found. The significance of the relatively small amounts of iron, which were consistently observed, is not clear. Spectrographic analysis confirmed the results of chemical analysis and failed to indicate the presence of significant amounts of other metals.

(e) *Substrate Specificity*—Y from pig liver is bleached most extensively by fatty acyl CoA derivatives of intermediate chain length (C_8 to C_{12}). It is only partially bleached by butyryl CoA and to even a lesser extent by palmityl CoA (Fig. 3). Y from pig liver is completely inactive in the "catalytic assay" with indophenol as acceptor unless supplemented with

[11] We are indebted to Dr. H. R. Mahler for a sample of chromatographically purified FAD and information about the tertiary butanol-water system.

ETF. This phenomenon will be more thoroughly discussed in the subsequent paper on ETF (8). When supplemented with ETF, Y shows its highest catalytic activity with fatty acyl CoA derivatives containing 8 to 12 carbons in the chain. A graphical presentation of the specificity of Y, Y', and G from pig liver was shown in a preliminary report (6). A more extensive account of the kinetics of the reactions catalyzed by the fatty acyl CoA dehydrogenases will appear in a subsequent communication.

(f) *Product of Dehydrogenation*—The product of the dehydrogenation of octanoyl CoA as catalyzed by Y and ETF was characterized by its further enzymatic reactions. In a way similar to that employed by Green *et al.* (2) with G it was shown that the oxidation product of octanoyl CoA is not capable of reducing DPN in the presence of the β-hydroxyacyl CoA dehydrogenase (20)[12] unless the unsaturated acyl CoA hydrase (crotonase)[12] (21, 22) is added. This suggests that the primary product of dehydrogenation is octenoyl CoA. It is most likely that the $\Delta^{2,3}$ isomer is formed.[2] In order to ascertain that the action of Y does indeed initiate a β oxidation the one-step oxidation of octanoyl CoA to octenoyl CoA was carried out. This compound, without being isolated, was then subjected to the action of the remaining enzymes of the fatty acid cycle in the presence of DPN and CoASH. Finally, malate, malic dehydrogenase, and the citrate condensing enzyme were added. Reduction of DPN occurred after this addition. Citrate was determined in the deproteinized reaction mixture. The following relationship was found: 0.43 μmole of dye was reduced[13] during the first oxidative step, 0.21 μmole of DPN was reduced on addition of malate, malic dehydrogenase, and condensing enzyme, and 0.23 μmole of citrate was found. The amount of indophenol originally added was such that the caproyl CoA formed by enzymatic cleavage of β-ketooctanoyl CoA could not be further oxidized. The agreement of the analytical values obtained is considered satisfactory in view of the complexity of the system. It may be concluded that a 2-carbon fragment is indeed split off from octanoyl CoA in the course of these reactions, or, in other words, that β oxidation takes place. Neither indophenol nor DPN was reduced and no citrate was formed, when Y and ETF were added after deproteinization.

Properties of Y from Beef Liver—An enzyme, very similar in its properties to Y from pig liver, had been isolated in high purity from beef liver before the existence of ETF and Y' was recognized. The presence of this enzyme in beef liver suggests that enzymes of the type of Y occur more generally in nature. On the other hand, the beef liver enzyme has some properties which distinguish it from the pig liver enzyme. A short account

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[12] Kindly provided by Dr. S. J. Wakil.

[13] Indophenol was used as oxidant, and a molar extinction coefficient of 16.1×10^6 sq. cm. $\times$ mole^{-1} was employed according to our determinations.

of the early work on the beef liver enzyme will therefore be given here, and the essential features are summarized in Table II together with those of the pig liver enzyme.

Since Y from beef liver was prepared before ETF and Y' were known, the preparative procedure used can no longer be considered entirely satis-

TABLE II

Comparison of Properties of Y Isolated from Pig and from Beef Liver

Property	Pig liver enzyme	Beef liver enzyme
Absorption maxima	275, 370, 447 mμ	275, 375, 445 mμ
Ratio of absorbance at these maxima	12.9:0.74:1.00	9.4:0.80:1.00
Decrease of absorbance on addition of substrate, about 0.3 μmole per ml.	At 447 mμ	At 445 mμ
Butyryl CoA	55%	57%
Octanoyl CoA	72%	63%
Palmityl CoA	35%	67%
Substrate of highest affinity*	C_{10}	C_{16}
Range of substrate specificity	C_4–C_{16}	C_4–C_{16}
Butyryl CoA in presence of palmityl CoA	Not oxidized	Not oxidized
Riboflavin content	0.22 ± 0.02%	0.28 ± 0.02%
Minimal molecular weight, riboflavin basis	160,000–190,000	125,000–145,000
Sedimentation coefficient,† $s_{20,w}$	7.66 ± 0.06 S at 10.3 mg. per ml.‡	7.7 ± 0.1 S at 8.2 mg. per ml.‡
Ratio, atoms of iron per molecule of flavin	1:6	1:3
Behavior in presence of 0.001 M Zn^{++} ions, pH 6.6§	Not pptd.	Not pptd.

* In this property Y from beef liver corresponds more closely to Y' rather than to Y from pig liver.

† A minimal molecular weight of about 140,000 and a probable molecular weight close to 200,000 can be calculated for both enzymes (see foot-note 9).

‡ Initial protein concentration.

§ Distinguishing characteristic between Y and Y' of pig liver.

factory. The following steps will, however, be of value in devising a satisfactory method of preparation. Y can be adsorbed on zinc hydroxide gel in a procedure similar to that described previously (2) and can be efficiently separated from remaining G and hemoproteins by fractionation with ethanol in the presence of 0.001 M Zn^{++} ions.

The purification was controlled by an assay which involved a direct titration of the flavoprotein. The reduction of the prosthetic flavin by substrate was measured spectrophotometrically. Since the enzyme is bleached

maximally by palmityl CoA, additional bleaching, when butyryl CoA is added after palmityl CoA, indicates the presence of G. This assay has the advantages of being rapid and insensitive to the presence of deacylating enzymes. On the basis of this assay, Y from beef liver was completely separated from G and was brought to an estimated purity of 60 to 70 per cent. This estimate was based on an ultracentrifuge experiment and on the spectral properties of Y. The enzyme was characterized by essentially the same methods as those employed for Y from pig liver.

The absorption spectrum is that of a flavoprotein. It has been presented in an earlier publication (23). The essential absorbance values and their ratios are reported in Table II. When the enzyme is reduced by substrate, the peaks at 445 and 375 mμ disappear. Y from beef liver, like the pig liver enzyme, shows the broad band at 500 to 650 mμ on reduction with substrate. As isolated, the enzyme seems to contain a hemoprotein impurity which is indicated by a shoulder in the Soret region.

In view of the minimal molecular weight indicated by the riboflavin content and the probable molecular weight calculated from the sedimentation coefficient, it is uncertain whether Y from beef liver has 1 or 2 flavins per molecule. The released prosthetic flavin is inseparable from FAD when chromatographed as described above. It also replaces FAD in the D-amino acid oxidase system.

At three levels of purification (purities of about 34, 45, and 67 per cent) iron was present in Y at a ratio of 1 atom of iron for 3 molecules of flavin. Copper was present at a ratio to flavin of 1:6. The copper content could be reduced to 20 per cent of this value by dialysis against cyanide without loss of catalytic activity with either indophenol or cytochrome c as acceptor. Arc spectroscopy[14] failed to indicate the presence of heavy metals other than copper and iron. The significance of the small amount of iron present is not clear.

The prosthetic flavin of the beef liver enzyme is bleached by fatty acyl derivatives of CoA from C_4 to C_{16}. Acetyl, propionyl, or isobutyryl CoA will not bleach Y. Isobutyryl CoA is the only substrate which bleaches G to some extent but does not bleach Y. This observation has been used as the basis of a differential assay.

Y from beef liver, at the stage of purity attained, catalyzed the oxidation of fatty acyl CoA derivatives from C_4 to C_{16} by indophenol. This ability to catalyze the over-all reaction was, however, lost on storage of Y in the frozen state for several months. Activity could be readily restored on addition of ETF. Fresh preparations of Y from beef liver, at all stages of purity, were stimulated about 3-fold in their catalytic activity by addition of ETF. On the other hand, side fractions were obtained from beef liver

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[14] We are indebted to Dr. V. W. Meloche of this university for these determinations.

preparations which had predominant activity as ETF. Such fractions were stimulated in their catalytic activity when supplemented with Y. It is therefore concluded that ETF occurs in beef liver and that Y from beef liver has the same requirement for ETF as electron carrier in the over-all catalytic process as does the pig liver enzyme. A certain amount of ETF, insufficient for optimal catalytic activity, seems, however, to accompany the beef liver enzyme very tenaciously through the purification. ETF then deteriorates preferentially on storage.

Although the beef liver enzyme shares with Y from pig liver a variety of properties, it differs from the pig liver enzyme in that its affinity for palmityl CoA is higher than that for octanoyl CoA. In this respect, the beef liver enzyme would correspond more closely to Y′ from pig liver. It is not known, and the matter was not further investigated, whether an additional enzyme corresponding in other respects to Y′ of pig liver is present in beef liver.

In the case of Y from beef liver K_s for palmityl CoA is of the order of 10^{-6} M. K_s increases with decreasing chain length of the substrate to 10^{-5} M for butyryl CoA. When the substrates of a carbon chain longer than C_6 are present at concentrations above 1 to 5×10^{-5} M, the over-all reaction catalyzed by Y and ETF is inhibited.

DISCUSSION

Among a great variety of procedures which have been tested, only a very few have proved successful in separating G, Y, Y′, and ETF. Green et al. (2) found zinc hydroxide gel unique in its ability to adsorb Y in preference to G in beef liver preparations. In the present work fractionation with zinc salts and ethanol (24), and this only after certain preliminary treatment, was the most successful procedure for separation of G, Y, Y′, and ETF on a preparative scale. It is of interest that even this step did not resolve G from Y in pig liver preparations. Zone electrophoresis on starch columns was the method of choice for the complete separation of sufficiently fractionated material. These observations are suggestive of a more than fortuitous association of the dehydrogenases and ETF. Such an association was further suggested by experiments on boundary electrophoresis. Unresolved preparations of Y and ETF frequently showed a high asymmetry of the pattern in the ascending and descending limb of the Tiselius apparatus.

The enzymes, which presumably function together under physiological conditions, may be released in one unit on disruption of the mitochondria. It is most likely that the preparations of the fatty acyl CoA dehydrogenases, whose properties have been described in earlier work, represented mixtures still partly unresolved.

Seubert and Lynen (25) have obtained a yellow flavoprotein from sheep

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ON THE MECHANISM OF DEHYDROGENATION OF FATTY ACYL DERIVATIVES OF COENZYME A

II. THE ELECTRON-TRANSFERRING FLAVOPROTEIN

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Paper I of this series (1) has dealt with a yellow flavoprotein (Y) which catalyzes the dehydrogenation of fatty acyl derivatives of CoA.[1] The prosthetic flavin of Y is reducible by substrate, but the reduced form (YH$_2$) is not oxidizable by conventional electron acceptors such as indophenol,[1] cytochrome c, ferricyanide, and oxygen except in the presence of a second flavoprotein (2) which acts as a link between YH$_2$ and these acceptors and which we have elected to call provisionally the electron-transferring flavoprotein (ETF; reduced, ETFH$_2$). The green flavoprotein, butyryl CoA dehydrogenase (G) (3), and a recently discovered yellow fatty acyl CoA dehydrogenase (Y'), specific for substrates of long carbon chain, are similarly dependent on ETF in their catalytic turnover.

The ability of ETFH$_2$ to react with cytochrome c is highly variable (2), in contrast to the consistent interaction with indophenol and ferricyanide. This variability can be accounted for in terms of protein interactions which occur in ETF preparations during fractionation and storage and which can be reversed under specific conditions. The present communication deals with the basic observations which led to the discovery of ETF and with the preparation and some of the characteristic properties of this enzyme. The basic phenomena related to the unusual interaction of ETF with cytochrome c as electron acceptor are also described.

EXPERIMENTAL

Materials and Methods—These are the same as those described in Paper I (1). The assays of ETF with the various electron acceptors are carried out as described for the yellow dehydrogenase Y (1), except that an excess

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[1] The following abbreviations are used: CoA, coenzyme A; DPN and TPN, di- and triphosphopyridine nucleotide, respectively; DPNH and TPNH, reduced DPN and TPN, respectively; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; indophenol, 2,6-dichlorophenolindophenol; Tris, tris(hydroxymethyl)aminomethane.

of ETF-free Y is added.   Butyryl CoA has been routinely used as the substrate for Y in these assays.   Units and specific activity are defined under these conditions in the same way as for Y.

Results

Discovery of ETF in Preparations of Y—When a purified preparation of Y from pig liver was subjected to electrophoresis in 0.2 M Tris acetate of pH 8.1, three major components were observed (Fig. 1).   The two pale yellow components of low mobility (Components I and II) were sampled jointly from the descending limb (cathode).   They showed no characteristics of Y.   A pure sample of the fast moving component (No. III) could
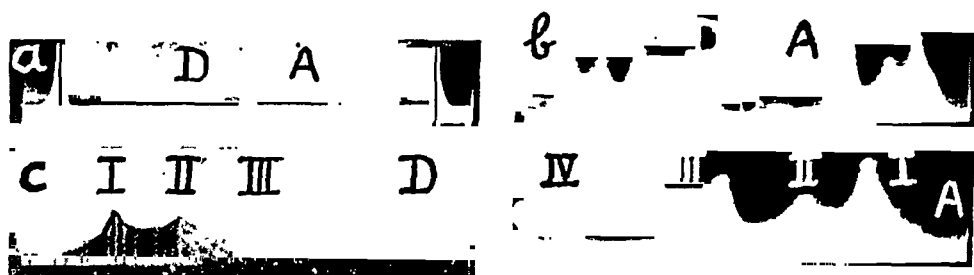
Fig. 1. Schlieren diagram of a purified pig liver preparation containing Y and ETF obtained in a Spinco model H electrophoresis unit.   40 mg. of protein were used in 0.2 M Tris acetate, pH 8.1, in the micro cell of the apparatus.   *A*, ascending limb (anode); *D*, descending limb (cathode).   Components I and II contain ETF, Component III represents Y of high purity, and Component IV a small amount of G.   Pictures were taken at 0 (*a*), 95 (*b*), and 183 (*c*) minutes.   The picture of the descending limb at 183 minutes was taken with the Hg line at 436 mμ and does not therefore record the deep yellow Component III.

be withdrawn from the ascending limb.   Component III, which was deep yellow, possessed the spectral properties of Y.   Its prosthetic flavin was readily reduced by octanoyl CoA.   Yet in the presence of Component III electron acceptors like indophenol or ferricyanide were not reduced by octanoyl CoA at a significant rate.   In other words, this flavoprotein with obvious characteristics of Y was not able to catalyze the dehydrogenation of octanoyl CoA by indophenol or ferricyanide, although it could undergo a stoichiometric oxidation-reduction with octanoyl CoA.   Recombination of this catalytically inactive Component III with Components I and II reconstituted the original catalytic activity of the material subjected to electrophoresis, as shown in Table I.   That the protein Component I or II or both Components I and II were necessary for this effect was demonstrated by the failure of various materials to substitute for them (Table I).   The active protein in Components I and II was found to be a flavoprotein which was called the electron-transferring flavoprotein (ETF).

The effect of ETF on other preparations of Y and on preparations of Y′ and G was then studied.   It was found that the catalytic activity of purified Y′ and G from pig liver and of all preparations of Y and G from beef liver with indophenol as acceptor was stimulated by ETF.

Preparation of ETF—The procedure is outlined in Table II.   All manipulations are carried out at 0–4° unless stated otherwise.   100 gm. of acetone powder of pig liver mitochondria are extracted with 1000 ml. of 0.02 M Tris acetate of pH 7.5 for 30 minutes.   The residue is removed by centrifugation.   20 ml. of 0.05 M zinc lactate of pH 5.5 are added slowly under stirring to every 100 ml. of the reddish brown extract, which contains 25

TABLE I

Restoration of Activity of Electrophoretically Separated Components

Component No	Indophenol reduced, $m\mu$moles per min. per mg. protein
Original sample.	60
I + II..	6
III	3
I + II + III	148*
III + 500 γ serum albumin	3
" + ashed (I + II)	3
" + deproteinized (I + II)	3
I + II + FAD	6

* This specific activity is calculated by subtracting the blank rate of Component I + II from the rate found in the presence of Component I + II + III and dividing by the amount of protein present in Component III.   Equal amounts of Component I + II and of Component III were combined.   The lower rate obtained with the original sample is due to the presence of the components in different proportions.

to 35 mg. of protein per ml. (specific activity 0.1 to 0.3).   The red precipitate is sedimented, and the supernatant fluid is brought to pH 7.2 and is then fractionated with solid ammonium sulfate into four fractions at 35, 50, 65, and 85 per cent saturation.   The fractions of 50 to 65 and 65 to 85 per cent saturation (specific activity 0.6 to 0.8) are dialyzed against 0.02 M Tris acetate of pH 7.5 until the ammonium sulfate concentration is reduced to less than 0.5 per cent.   The protein concentration is adjusted to about 20 mg. per ml., and 20 ml. of 0.05 M zinc lactate of pH 5.5 are added as above per 100 ml. of enzyme solution.   The precipitate is removed by centrifugation, and the supernatant fluid is fractionated with ethanol without prior pH adjustment into three fractions at 10, 16, and 40 per cent ethanol content.   The temperature is gradually lowered to −15° during this fractionation.   The precipitated zinc-protein complexes are

taken up in 0.4 M citrate of pH 7.2, and the fractions are dialyzed for 2 to 3 hours against 0.02 M Tris acetate of pH 7.2. The first ethanol fraction (specific activity 0.1) contains most of the Y and may be used for further purification of this enzyme. The third ethanol fraction (16 to 40 per cent; specific activity 1.3 to 1.5) is yellow and contains most of the ETF.

This material is then further fractionated with solid ammonium sulfate at a protein concentration of about 20 mg. per ml. without adjustment of pH. Fractions are collected at 50, 55, 65, 90, and 100 per cent saturation. ETF of highest activity is found in the fraction between 65 and 90 per cent ammonium sulfate (specific activity 1.7). Although little purification is

TABLE II

Purification of ETF

Fractionation step	Protein	Specific activity*	Units	Recovery
	mg.			*per cent*
Extraction of 350 gm. acetone powder of pig liver mitochondria...................................	66,500	0.12	8000	100
Zn^{++}-ammonium sulfate (50–65 and 65–85%)........	7,560	0.58	4400	55
Zn^{++}-ethanol (16–40%).............................	840	1.4	1180	14.7
Ammonium sulfate, pH 6.5–7 (65–90%).............	245	1.7	416	5.2
" " " 8.1 (70–75%)............ ...	18	3.6	64	0.8
" " " 8.1 (75–80%)...........	21	5.5	118	1.5
" " " 8.1 (80–100%)...............	14	4.3	60	0.75

* Specific activity with indophenol as acceptor defined as the decrease in optical density at 600 mμ per minute and per mg. of protein in 0.5 ml. volume.

achieved in this step, it seems necessary for the successful outcome of the following step.

The most active material from the preceding step is dialyzed against 0.2 M Tris acetate of pH 8.1. The dialyzed solution is diluted to a protein concentration of 15 to 20 mg. per ml. with the same buffer. This solution is then fractionated with solid ammonium sulfate with no further adjustment of pH, which in this case remains constant at 8.1 throughout the fractionation. Fractions are collected at 60, 65, 70, 75, 80, and 100 per cent saturation. The material of highest activity is obtained at 75 to 80 per cent saturation (specific activity 5 to 6). The adjoining fractions show also substantial purification and contain about half of the recovered units. As in the procedures used for the purification of the yellow and green dehydrogenases, which are closely associated with ETF, satisfactory separation of the related proteins was only achieved by a considerable sacrifice in yield.

Purity Criteria—Since ETF is a flavoprotein, the ratio of the absorbance at the ultraviolet absorption maximum at 270 mμ or the minimum at 310 mμ to that at the absorption maximum in the visible region at 437.5 mμ can serve as a criterion of purity. These spectral properties will be discussed below.

A preparation of ETF with a value of 7.9 for $E_{270}:E_{437.5}$ was studied during boundary electrophoresis in 0.2 M Tris acetate of pH 8.1 (Fig. 2). ETF is stationary under these conditions. This is in agreement with the results obtained on crude preparations, as shown in Fig. 1. Although no material with an essentially different mobility was observed, the boundaries showed
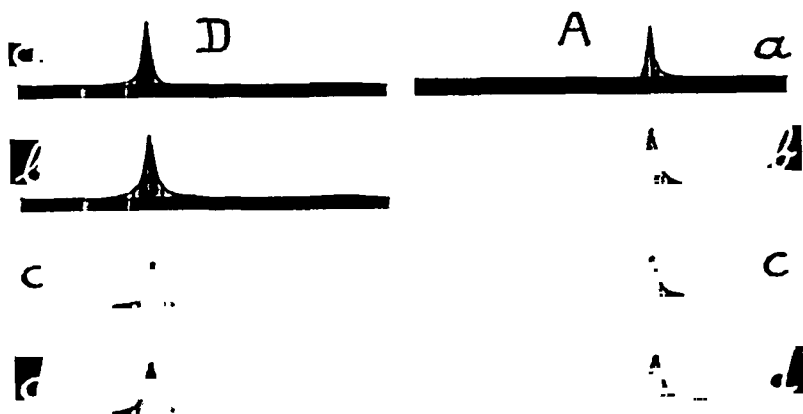
Fig. 2. Schlieren diagram of ETF obtained in a Spinco model H electrophoresis unit. 11 mg. of protein were used in 0.2 M Tris acetate, pH 8.1, in the micro cell of the apparatus. *A*, ascending limb (anode); *D*, descending limb. Pictures were taken at 0 (*a*), 73 (*b*), 155 (*c*), and 248 (*d*) minutes.

definite asymmetry after $2\frac{1}{2}$ hours and more so after 4 hours, indicating the presence of more than one component.

An analogous preparation of the same spectral properties was studied in the analytical ultracentrifuge (Fig. 3). A single boundary was observed which sedimented with the yellow color. This boundary remained symmetrical during the $1\frac{1}{2}$ hours of the experiment. The sedimentation coefficient corrected to water at 20° was calculated[2] to be $s_{20,w} = 3.86 \pm 0.04$ S at the protein concentration used (see the legend to Fig. 3). It is estimated that ETF is likely to have a molecular weight of 30,000 to 70,000.

Preparations of ETF, after the Zn^{++}-ethanol treatment, are completely free of the two yellow dehydrogenases, of G, and of other enzymes of the

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[2] The formula $s_{20,w} = s_{experimental} \times \eta_{experimental}/\eta_{20,w}$ was applied for this correction.

fatty acid cycle, but preparations of even the highest purity contain variable amounts of another flavoprotein which catalyzes the oxidation of DPNH and TPNH by indophenol or cytochrome c (under certain conditions). The relationship of this diaphorase-like enzyme to ETF is not yet clear. If it is a flavoprotein of the high catalytic activity of the known diaphorases and cytochrome reductases, a 1 to 5 per cent contamination of ETF with such an enzyme could account for the observed activity. Studies with inhibitors, the pH-activity relationship, and the fact that the activity towards DPNH is inseparable from that towards TPNH indicate, however, that the diaphorase or cytochrome reductase in the ETF preparations is not identical with similar ones described in the literature (4–6).

Absorption Spectrum of ETF—All highly active preparations of ETF are deep yellow and exhibit a characteristic absorption spectrum (Fig. 4).
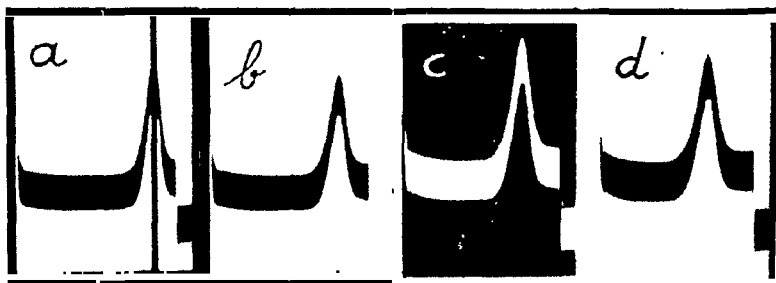
FIG. 3. Schlieren diagram of ETF in a Spinco model E analytical ultracentrifuge (mean temperature, 9.3°; rotor speed, 59,780 r.p.m.; buffer, 0.05 M potassium phosphate, pH 7.5; initial protein concentration, 14.4 mg. per ml.). Pictures taken at 46 (*a*), 62 (*b*), 78 (*c*), and 94 (*d*) minutes.

Basically it is that of a flavoprotein with peaks at 270, 375, and 437.5 mμ, but it has in addition some features unusual for flavoproteins. There is an additional minor peak at 460 mμ which invariably appears as a distinct shoulder in all ETF preparations, even those of low purity. It is of interest that a less pronounced shoulder in the corresponding spectral region has been observed with L-amino acid oxidase (7), the old and new yellow enzymes (8), and Straub's diaphorase (4). Furthermore, the extremely low values of 6.5 for $E_{270}:E_{437.5}$ and 0.3 for $E_{310}:E_{437.5}$ which have been obtained in ETF preparations are also unusual and are the lowest recorded for flavoproteins. Such spectral ratios would seem to make it quite unlikely that there is much contamination by colorless proteins in these preparations. There is, however, evidence that at least one more flavoprotein is present in these preparations, as discussed above. There is also in the spectra of all preparations of ETF a shoulder at about 410 mμ. It appears as a distinct peak at 415 mμ in preparations from which part of the flavin has been removed by precipitation with acid ammonium sulfate.

This shoulder in the Soret region may be an indication of a contaminating hemoprotein. The significance of the presence of this hemoprotein is not yet apparent.
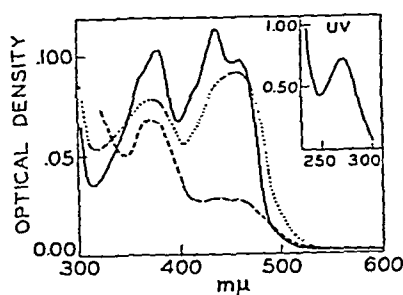
FIG. 4. Absorption spectrum of ETF. 180 γ of protein were dissolved in 0.4 ml. of 0.025 M phosphate, pH 7.0. Solid line, oxidized form; dash line, reduced with 0.03 μmole of palmityl CoA and 15 γ of Y; corrected for absorption of Y in the presence of 0.03 μmole of palmityl CoA. Dotted line, spectrum of flavin released from ETF by acid; spectrum taken after neutralization and corrected to the same volume in which ETF (as shown by the solid line) was originally present.

TABLE III

Reactivation of Split ETF* with FAD†

Preparation No.	Acceptor indophenol, specific activity‡				Acceptor cytochrome c, specific activity‡			
	Before splitting	Split enzyme			Before splitting	Split enzyme		
		Without FAD	With FAD	With FMN		Without FAD	With FAD	With FMN
1	6.7	5.5	6.5		0.55	0.64	4.6	
2					0.48	1.2	4.0	
3	8.6	2.5	3.6	2.6	1.1	2.3	4.1	2.3

* In the assays for enzymatic activity ETF was reduced with butyryl CoA and Y.
† FAD and FMN were added to a final concentration of 2.5×10^{-5} M.
‡ For specific activity with indophenol see Table II; specific activity with cytochrome c is defined in the same way as that for indophenol except that the increase in optical density at 550 mμ is substituted for the decrease at 600 mμ.

ETF preparations of high purity have a very distinct greenish fluorescence.

Prosthetic Group—When ETF is precipitated with ammonium sulfate at pH 4.2, up to 80 per cent of the prosthetic flavin may be detached from the enzyme. Concomitant with the loss of flavin, enzymatic activity is lost, which can be restored by addition of FAD but not of FMN (Table III). It is remarkable that a more pronounced stimulation is consistently

observed when cytochrome c is the acceptor rather than indophenol. With cytochrome as acceptor, a tentative dissociation constant for FAD was derived by measurements at various FAD concentrations and evaluation by the conventional plot (9). The apparent K_D was found to be of the order of 10^{-5} M in this case. When the prosthetic flavin is released from ETF by treatment with trichloroacetic acid, the spectrum indicated by the dotted line in Fig. 4 is obtained. The value of $E_{260}:E_{450}$ of the detached flavin is higher than theoretical for FAD (3.3) (10, 11) unless the flavin is further purified.

The released flavin was subjected to chromatography on paper in the two systems described in Paper I. It showed the same R_F values as pure FAD and was inseparable from added FAD. The flavin from ETF was also tested for its ability to replace FAD with D-amino acid apooxidase prepared from pig kidney by the method of Negelein and Brömel (12). Values were obtained at various flavin concentrations and evaluated by the conventional reciprocal plot (9). Under the conditions used, the apparent K_D for FAD was found to be 3.3×10^{-7} M and for the flavin of ETF 3.7×10^{-7} M. These experiments indicate that FAD is the prosthetic group of ETF. A preparation of ETF with a value of 6.5 for $E_{270}:E_{437.5}$ was precipitated in 0.5 per cent perchloric acid and treated as described previously (1). The FAD content of ETF was 0.94 per cent and the riboflavin content 0.45 per cent. This would correspond to a minimal molecular weight of 83,500. Since a molecular weight range of 30,000 to 70,000 was indicated by the ultracentrifuge, it appears most likely that ETF has a molecular weight of about 80,000 and contains 1 flavin per molecule.

Spectrographic analysis for heavy metals indicated the presence of only traces of iron and copper.[3] According to chemical determinations, there was less than 1 atom of iron present per 5 moles of flavin. Copper was present at an even smaller ratio to flavin. Molybdenum was absent. The significance of the small amount of iron found is doubtful.

Interaction of ETF with Dehydrogenases—When a fatty acyl CoA is added to ETF, no change occurs in the spectrum. Then, when in addition to the CoA derivative a catalytic amount of Y is added, ETF is reduced in a few minutes as indicated by the dash line in Fig. 4. The absorption at 437.5 mμ drops to 25 per cent of its original value. Addition of dithionite leads to a further small reduction of the residual absorption. G or Y' can fulfil the same function that Y does in such experiments provided a fatty acyl CoA of an appropriate carbon chain is added. Experiments of this type constitute convincing proof that the sequence of events in the dehydrogenation of fatty acyl derivatives of CoA is the following (see the scheme

in "Discussion"): the electrons are passed from the CoA derivative to Y, Y', or G and then to ETF. It was of interest to determine whether the oxidation-reduction between YH₂, Y'H₂ or GH₂, and ETF could be readily reversed, *i.e.* whether ETFH₂ could reduce Y, Y', or G. In an approach to this problem advantage was taken of the fact that Y, but not G, is bleached by palmityl CoA. Y and ETF were therefore reduced with an excess of palmityl CoA, and G was added after the reduction of Y and ETF was complete. No reduction of G could be observed when ETFH₂ and G were present in approximately equimolar amounts with respect to their prosthetic flavin, a condition necessary for proper spectrophotometric measurement. Apparently, the equilibrium in such a system is sufficiently in favor of ETFH₂ so that a reversal cannot be detected.
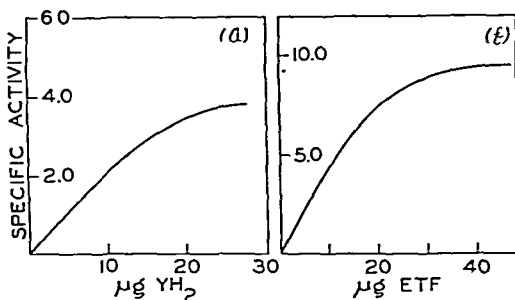
Fig. 5. (a) Dependence of rate of indophenol reduction by ETFH₂ on the concentration of substrate if YH₂ is considered the substrate of ETF. Standard conditions of indophenol assay with 47 γ of ETF (specific activity 6.0). 0.05 μmole of butyryl CoA added after Y and ETF had been incubated for 1 minute. (b) Dependence of rate of indophenol reduction by YH₂, as catalyzed by ETF, on the concentration of ETF. Conditions as in (a), except that the fixed amount of 14 γ of Y (specific activity 11.7) was present and varied amounts of ETF.

The relationship between ETF and Y in the over-all oxidation of fatty acyl derivatives of CoA by indophenol is shown in Fig. 5, *a* and *b*. In the experiments of Fig. 5, *a* YH₂, formed from Y by the presence of an excess of butyryl CoA, was treated as the substrate of ETF; in the experiments of Fig. 5, *b* ETF was treated as the electron-accepting substrate of YH₂. In both cases, which admittedly represent crude first approximations, Michaelis-Menten behavior was satisfactorily followed. Approximate K_s values, based on the molarity of flavin in the enzymes, were derived from these experiments. K_s for YH₂ as substrate of ETF was found to be of the order of 10^{-6} M. A K_s of 10^{-7} M was found for GH₂ as substrate of ETF. K_s for ETF as substrate of YH₂ or GH₂ was of the order of 10^{-6} M. The high affinity of the components of this flavoprotein system for each other is indicated.

Electron Acceptors—Although ETF has so far been found to be specific

for its electron-donating substrates, *viz.* YH₂, Y'H₂, and GH₂, it can react with a variety of electron acceptors. Most of the present work was carried out with indophenol, but electrons can be readily transferred from ETFH₂ to ferricyanide, cytochrome *c*, many quinones, and *N*-methylphenazine. The last two types of acceptors have a limited ability to interact with YH₂, Y'H₂, or GH₂ directly (1). YH₂, Y'H₂, and GH₂ are not oxidized by oxygen when substrate is present, and ETFH₂ is only slowly autoxidizable.

Interaction of ETF with Cytochrome c—Considerable attention was initially given to the interaction of ETFH₂ with cytochrome *c*, because it was surmised that the natural function of ETF was to link the dehydrogenation of fatty acyl derivatives of CoA to cytochrome *c*. As pointed out earlier, the ability of ETFH₂ to interact with cytochrome *c* was variable. Such ability was only found in certain ETF fractions and was largely lost on a few days storage in the frozen state without any perceptible change in the spectrum. It was first thought (2) that a component necessary for interaction with cytochrome *c* was lost or had undergone some change in state in the isolated enzyme. This seemed the more likely, as similar observations had previously been made with DPNH cytochrome reductase (13). In this case the loss of iron accounted for the loss of cytochrome *c*-reducing activity, and this activity could be partially restored by addition of iron. A great variety of additions of cofactors, metal salts, boiled and concentrated extracts, extracts of ashed material, and protective proteins neither stabilized nor restored the cytochrome *c*-reducing activity of ETFH₂. An explanation was finally provided by experiments on acid ammonium sulfate treatment of ETF, which were carried out to detach the prosthetic flavin. After this treatment the ability of ETFH₂ to reduce cytochrome *c* was increased up to 6-fold,[4] whereas the ability to reduce indophenol was at most doubled and in most cases actually diminished. It is evident from the data presented in Table IV that there was not only an increase in specific activity, but also a substantial increase of the total units originally present. The recovery of indophenol units indicates that the occasional increase in specific activity with indophenol is merely due to further purification of ETF. The drastic change is specifically evident from the last columns of Table IV, where the ratio of indophenol to cytochrome units before and after acid treatment is compared. These observations are interpreted to

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[4] It was shown under "Prosthetic group" that FAD is released from ETF under the conditions used here. The quantity of FAD released varies from preparation to preparation. The activity of acid-treated preparations is therefore stimulated to a variable extent by added FAD. Activation of the ability to reduce cytochrome *c* was generally observed whether FAD was added or not (see Table III). In many cases the activity was higher in the presence of FAD. FAD was therefore routinely added in assays of acid-treated fractions in order to establish comparable conditions. It should be pointed out, however, that the activation phenomenon as such did not depend on the addition of FAD.

mean that the cytochrome c-reducing rate of ETFH$_2$ declines not because some essential component is lost, but because an active site in ETF is blocked which can be exposed again by acid treatment. As the data for Preparation 1 in Table V show, the activation by acid treatment is to a certain extent reversible, since the rate of cytochrome c reduction by ETFH$_2$ declines again when the treated material is stored for a short time.

The rate of ferricyanide reduction by ETFH$_2$ is not influenced by all these treatments, but is as stable as the rate of indophenol reduction. The fact that the 1-electron acceptor ferricyanide acts like indophenol in this case suggests that the peculiar behavior of ETFH$_2$ towards cytochrome c as acceptor is completely unrelated to the requirement of a 1-electron transfer in the reaction with cytochrome c. Evidence had been presented by

TABLE IV

Activation and Decline of Cytochrome-Reducing Ability of ETFH$_2$*

Preparation No.	Acceptor indophenol						Acceptor cytochrome c						Indophenol units Cytochrome units	
	Specific activity†			Units			Specific activity†			Units				
	(a)‡	(b)‡	(c)‡	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)
1	2.8	6.4		5.0	4.8		0.5	3.3		0.9	2.5		5.5	1.9
2	5.5	3.8		66	36.5		1.0	3.8		11.5	36.5		5.7	1.0
3	8.6	5.1	3.64	19.5	7.9	5.6	1.1	4.5	1.73	2.5	7.0	2.7	7.8	1.1
4							0.13	0.30	0.14	3.2	5.7	2.7		

* ETF was reduced with butyryl CoA and Y.

† See Tables II and III.

‡ (a) Before, (b) immediately after treatment with ammonium sulfate at pH 4.2, (c) after treatment and storage of 1 week for Preparation 3 and 1 day for Preparation 4.

Mahler *et al.* (14) and Mackler *et al.* (15) that the difference in the interaction of other flavoproteins with 2-electron dyes like indophenol, on the one hand, and cytochrome c, on the other, could be explained by the requirement of a metal for the 1-electron transfer to cytochrome c, whereas a 2-electron transfer could proceed without metal. In the present case of the interaction of ETFH$_2$ with cytochrome c, no metal was found to be implicated, and the observations reported above are best explained by the assumption that specific spatial requirements have to be fulfilled before the reaction of ETFH$_2$ with cytochrome c can take place.

Fractionation experiments indicate that the interaction of ETF with cytochrome c is interfered with by a protein which is present in ETF preparations. This protein can be partly separated from ETF (Table V). It is noteworthy that such separated inhibitory fractions are particularly rich in the diaphorase which occurs in ETF preparations (see "Purity cri-

teria" above).　It is also remarkable that this diaphorase, when associated with ETF, undergoes similar changes in ability to react with cytochrome c on treatment with acid ammonium sulfate or on storage.

TABLE V

Decline and Inhibition of Cytochrome-Reducing Ability of ETFH₂ and of Associated Dihydropyridine Nucleotide Dehydrogenase

Prepara-tion No.	Eluate of ammonium sulfate ppt. at pH 4.2	Acceptor				Specific activity with indophenol / Specific activity with cytochrome c	
		Indophenol		Cytochrome c			
		Substrate				YH₂	DPNH
		YH₂*	DPNH	YH₂	DPNH		
		Specific activity†					
1	Eluate immediately after elution	1.1	5.0	1.5	6.5	0.73	0.77
	Eluate 2 days after elution	1.7	6.7	0.75	0.17	2.3	39
2	Before acid treatment	6.3	8.6	0.46	0	13.7	∞
	Eluate 2 of ppt. at pH 4.2	0.30	3.3	0.53	0.03	0.56	110
	"　5　"　"　"　" 4.2	0.75	6.7	0.75	0.4	1	17
	"　2 (17 γ protein) + Eluate 5 (4 γ protein)	0.6	10	0.06	0	10	∞
3	Eluate 2	2.8	3.2	1.7	0	1.65	∞
	"　4	0.35	2.7	0.05	4.0	7	0.7
	"　2 (19 γ protein) + Eluate 4 (37 γ protein)	3.9	3.2	0.95	0.36	4.1	9

The protein precipitate obtained at pH 4.2 at a saturation of 95 per cent with respect to ammonium sulfate was eluted with 0.02 M Tris acetate, pH 7.5.　With Preparation 1 a single eluate was obtained with 0.03 ml. of buffer per mg. of protein precipitate.　With Preparations 2 and 3 successive eluates were made with about 0.01 ml. of buffer per mg. of protein.　The specific activity of the recombined eluates was computed by subtracting the blank rate of the inhibitory fraction from the observed rate and dividing this corrected rate by the protein value of the inhibited fraction.　The later eluate (No. 4 or 5) is always the inhibitor of YH₂ oxidation and the earlier eluate (No. 2) is the inhibitor of DPNH oxidation with cytochrome c as acceptor.
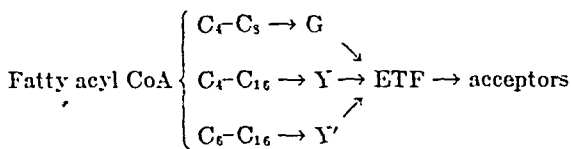
* Y was reduced with butyryl CoA.

† See Tables II and III.

DISCUSSION

The present investigation has established the principle that flavoproteins may operate in series in hydrogen or electron transfer.　The accompanying scheme represents our present picture of hydrogen or electron

transport in the enzymatic dehydrogenation of fatty acyl derivatives of CoA. The mechanism by which an oxidation-reduction can take place between proteins which have tightly bound prosthetic groups[5] deserves interest. A direct oxidation-reduction between enzymes, *viz.* between triosephosphate dehydrogenase and lactic dehydrogenase (16) or triosephosphate dehydrogenase and DPNH cytochrome *c* reductase (17), had previously been observed. However, it is simpler to conceive of a mechanism in these two instances, because it can be visualized that the bound prosthetic group (DPN) of triosephosphate dehydrogenase can be shared by the other enzymes which have a binding site for readily dissociable DPN.

$$\text{Fatty acyl CoA} \begin{cases} C_4\text{-}C_8 \rightarrow G \\ \\ C_4\text{-}C_{16} \rightarrow Y \rightarrow ETF \rightarrow \text{acceptors} \\ \\ C_6\text{-}C_{16} \rightarrow Y' \end{cases}$$

As far as our present knowledge goes, ETF is specific for its electron donors, *viz.* YH_2, $Y'H_2$, and GH_2. The question is raised, however, whether other electron carriers of this type might not occur more generally in electron transfer systems. It seems possible that some of the more complex dehydrogenases of the flavoprotein group may eventually be resolved into a primary dehydrogenase and an electron carrier moiety analogous to Y and ETF.

The principle of a coupled flavoprotein system has also some practical implications which have become obvious in the description of the initial difficulties of the work reported in this and in Paper I. It may be misleading to follow enzyme purification by measurement of catalytic activity with an arbitrarily chosen electron acceptor. A similar conclusion has been recently arrived at by Singer and Kearney (18) who showed that succinic dehydrogenase could be readily purified as a soluble enzyme only if a specific electron acceptor was used to follow its activity. One may therefore suspect that the difficulties encountered in the purification of certain other enzymes will eventually be explained along similar lines. A second practical implication of the direct oxidation-reduction between flavoproteins concerns the reducibility of the prosthetic group by substrate. The reduction of the prosthetic group of a flavin enzyme by its substrate is frequently used as a reliable and convenient means of identification. The fact established in the present paper that one flavoprotein may reduce another obviously calls for caution when quantitative information is to be derived

[5] Although the apparent dissociation constant for FAD reported above was relatively high after ETF had been split with acid ammonium sulfate, there is no evidence that the native enzyme releases FAD under any conditions or is stimulated in the presence of FAD.

from the extent of bleaching by substrate. On the other hand, it has been shown in Paper I that the complex of a reduced flavoprotein with the oxidized substrate may absorb light in the spectral region of the flavin peaks and may thus likewise interfere with a quantitative evaluation of absorbance changes in the flavin component proper.

New interest is focused by the present work on the actual rôle of copper in G. ETF was found necessary for optimal interaction of G with indophenol as well as with cytochrome c. If ETF intervenes in electron transfer between G and cytochrome c, the pathway of electron transport in this system is certainly more complex than that originally proposed (19). It is possible that a slow direct reaction of G with cytochrome c may be dependent on the presence of copper in the way suggested earlier (19). However, it cannot be decided at the moment whether such small residual catalytic activity with indophenol or cytochrome c, as is occasionally present in preparations of G or Y isolated from beef liver, is due to contamination with ETF or to an intrinsic ability of these enzymes to react directly with acceptors to a limited extent. The very fact that this ability is only found occasionally and is lost on prolonged storage would make it more likely that contamination by ETF is indeed the explanation for this residual activity.

The rôle of cytochrome c as electron acceptor in the dehydrogenation of fatty acyl derivatives of CoA requires some comment. The strange situation has been encountered that the ability of a flavoprotein to interact with cytochrome c can be increased several times by artificial means. These observations cast doubt on the actual significance of cytochrome c as electron acceptor in these systems in their natural state. How far these considerations apply to other systems which can be linked to cytochrome c remains to be determined.

SUMMARY

The preparation and some properties of a new flavoprotein (ETF) which specifically catalyzes the oxidation of the reduced forms of the green and the yellow fatty acyl CoA dehydrogenases by indophenol, ferricyanide, or cytochrome c are described. The prosthetic group of ETF appears to be FAD. The riboflavin content of ETF is 0.45 per cent, and its minimal molecular weight is 83,500 according to this flavin content. The ability of ETF to interact with cytochrome c is very variable, in contrast to the consistent interaction with other electron acceptors. This variability can be accounted for in terms of protein interactions which occur in ETF preparations and which are reversed under specific conditions. The implications of the present work are discussed with respect to flavoprotein catalysis and electron transfer in general and with respect to the significance of

cytochrome c as natural electron acceptor in the present system and the rôle of copper in butyryl CoA dehydrogenase.

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A STUDY OF THE TRANSAMINATION REACTION BY USE OF ISOTOPIC NITROGEN*

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The general nature of the biological transamination reaction (1) has been amply demonstrated in the past few years (2–7). It has been suggested (8) that the coenzyme, via alternate interconversions into pyridoxal and pyridoxamine phosphates, functions as the shuttle of the amino groups in these reactions. If this mechanism is valid, it then follows that enzymatic transaminations between the coenzyme and α-amino or α-keto acids should be as wide-spread as between the amino acid pairs themselves. As an instance of this, we have studied the enzymatic transaminations between α-ketoglutarate and N^{15}-labeled pyridoxamine in a pig heart homogenate. The validity of this approach for an investigation of the mechanism of the transamination reaction was first tested in experiments in which N^{15}-labeled amino acids were the amino donors to α-ketoglutarate.

EXPERIMENTAL

Synthesis of Substrates—α-Ketoglutaric acid (m.p. 112–113°) was made by the condensation of diethyl succinate with diethyl oxalate according to the procedure of Friedman and Kosower (9). L-Serine-N^{15}, $[\alpha]_D^{23}$ $-6.8°$ (4.4 per cent in water), was obtained by resolution (10) of synthetic racemic serine (11). DL-Valine-N^{15}, DL-leucine-N^{15}, and glycine-N^{15} were prepared by the method of Schoenheimer and Ratner (12). By taking advantage of the differential solubility (13) of the L- and DL-leucine 2-bromotoluene-5-sulfonates in 1 N hydrochloric acid, L-leucine-N^{15} was obtained by isotopic dilution of the DL-leucine-N^{15}.

20 gm. of non-isotopic L-leucine and 2 gm. of DL-leucine-N^{15} of approximately 32 atom per cent excess were dissolved in 142 ml. of hot 1 N hydrochloric acid and precipitated with 2-bromotoluene-5-sulfonic acid. The salt was recrystallized twice from 200 ml. of 1 N hydrochloric acid, a volume

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of solvent easily sufficient to keep the DL-leucine salt in solution. After decomposition of the precipitate with barium acetate, L-leucine was isolated in the usual manner (14); yield, 12.2 gm. of L-leucine; $[\alpha]_D^{22}$ +15.0° (2.2 per cent in 6 N hydrochloric acid). Lysine-2-N¹⁵, histidine-2-N¹⁵, and phenylalanine-N¹⁵ were preparations previously made in this laboratory (15, 16).

Isotopic Pyridoxamine—The procedures in the literature (17) for the preparation of pyridoxamine are unsuited for adaptation to an isotopic

$$KN^{15}O_3 + Pb \longrightarrow KN^{15}O_2 + PbO$$

$$KN^{15}O_2 + NaHSO_3 + SO_2 \longrightarrow HON^{15} \Big\langle {}^{SO_3Na}_{SO_3K}$$

$$\xrightarrow[(CH_3)_2C=O]{} (CH_3)_2C = N^{15}OH \xrightarrow[H_2O]{HCl} H_2N^{15}OH \cdot HCl$$
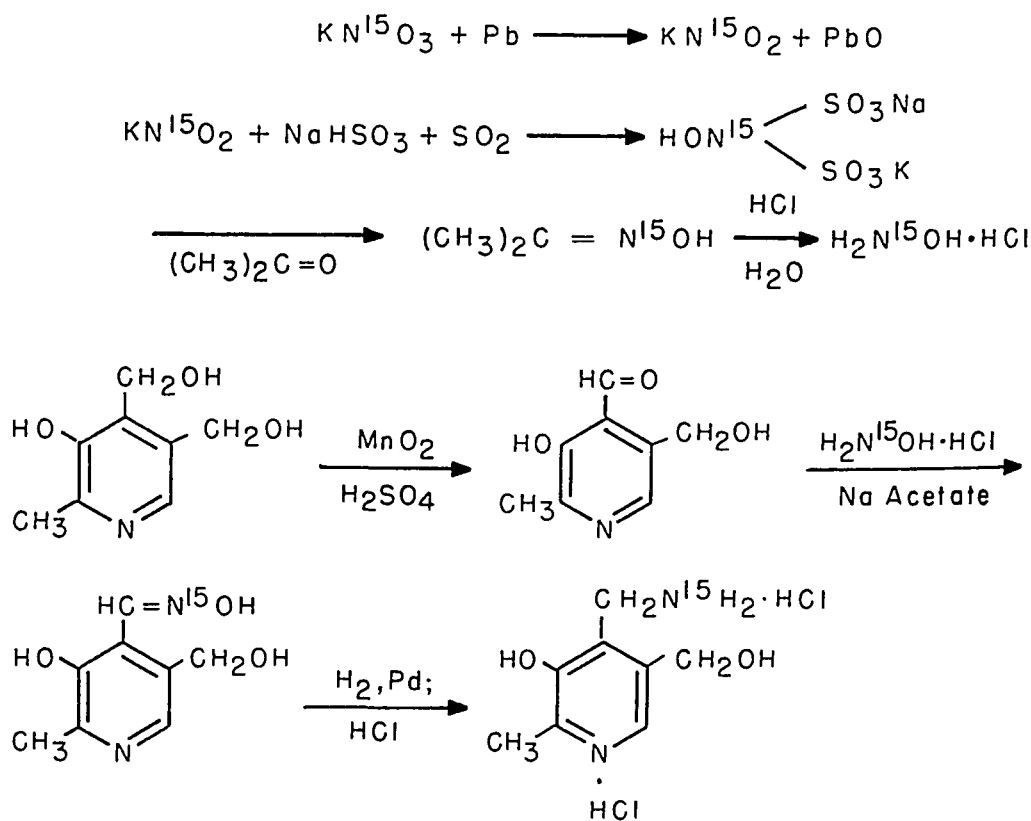
FIG. 1. Isotopic synthesis of pyridoxamine

synthesis, since they require the use of excess aqueous or liquid ammonia. It was found that catalytic reduction (18) of the oxime under the proper conditions gave an excellent yield of pyridoxamine. This procedure necessitated the synthesis of isotopic hydroxylamine, so that the nitrogen isotope could be introduced into the 4-aminomethyl position (see Fig. 1).

KN¹⁵O₃ (obtained from Eastman Kodak) was dissolved and reduced with lead powder according to the directions of Vanino (19). Trial experiments on this conversion averaged an 80 per cent yield of nitrite. The aqueous solution of isotopic KNO₂ was then reduced to hydroxylamine sulfate with SO₂, trapped as acetoxime, and purified and isolated as the hydrochloride (20); yield, 16 per cent (based upon KNO₃); m.p., 145°; N¹⁵

concentration, 13.98 atom per cent excess.   The isotopic hydroxylamine hydrochloride gave an immediate, positive Benedict test at room temperature.   When this compound was heated in water with sodium acetate and benzoyl chloride, it gave the characteristic color for benzhydroxamic acid.

The hydroxylamine hydrochloride (3.06 gm.) was coupled to pyridoxal made from pyridoxine (0.05 mole), and pyridoxal oxime, weighing 3.64 gm. (39 per cent yield based upon $H_2NOH \cdot HCl$), m.p. 229–230°, was obtained (21).   A second isotopic batch prepared in this fashion was obtained in 50 per cent yield.

20 mmoles of pyridoxal oxime were dissolved in 150 ml. of glacial acetic acid which had been previously distilled over potassium permanganate. Freshly prepared palladium black catalyst in glacial acetic acid was added to the solution containing the oxime.   Reduction was allowed to proceed at slightly greater than atmospheric pressure.   After 3 hours the theoretical amount of hydrogen had been taken up.   The catalyst was filtered, 20 ml. of 6 N hydrochloric acid were added to the filtrate, and the acetic acid was removed by distillation *in vacuo*.   The remaining syrup was taken up in 50 ml. of 95 per cent alcohol and was saturated with hydrochloric acid gas in the cold.   After standing for 5 minutes in an ice bath, white crystals of pyridoxamine dihydrochloride began to appear.   A small amount of ether was added to complete the precipitation.   After refrigeration overnight the product was filtered and washed with ether.   It was dissolved in 60 ml. of hot alcohol and reprecipitated as above.   After collection and drying *in vacuo* at 63°, 3.04 gm. (65 per cent yield) of product, m.p. 221–224° and mixed m.p. with an authentic sample 219–222°, were obtained.   Its N^{15} concentration was 1.42 atom per cent excess.   The reduction of a second batch of pyridoxal oxime afforded a 77 per cent yield of pyridoxamine dihydrochloride taken as starting material.   The melting point of this preparation was 219–220°, neutral equivalent 126, and N^{15} concentration 1.31 atom per cent excess.   The calculated neutral equivalent is 120.5.   The absorption spectrum of this compound was identical with that of an authentic sample.   5 mg. of this preparation dissolved in 4 ml. of Nessler's reagent gave a negative test for ammonia.

A portion of the pyridoxamine dihydrochloride prepared as outlined above was converted to the free base by treatment with sodium bicarbonate in water.   The resulting precipitate was centrifuged in the cold and recrystallized from water, m.p. 186–188° and mixed m.p. 185–186° with an authentic sample.   Upon solution in hot alcohol and treatment with a

$C_8H_{12}O_2N_2$ (168.2).   Calculated.   C 57.1, H 7.2, N 16.6
                                         Found.   " 57.2, " 7.1, " 16.1

saturated alcoholic picric acid solution, the synthetic pyridoxamine readily

gave the dipicrate. Upon recrystallization from alcohol, the derivative melted sharply at 200° (decomposition).

Enzymatic Transaminations—Fresh pig hearts were obtained and immediately frozen with dry ice. 60 gm. of thawed muscle tissue were homogenized in a Waring blendor for 5 minutes with 300 ml. of 0.125 per cent potassium bicarbonate solution (1). The isotopic substrate, α-ketoglutaric acid, and ammonium chloride were then added to the homogenate (see Tables I to IV for protocols), and the pH was adjusted to 7.4 to 7.5 by the addition of solid potassium bicarbonate. Incubation was carried out for 1 hour at 37° with shaking. At the end of this period, solid trichloroacetic acid was added to a final concentration of 6 to 8 per cent. The precipitated protein was filtered to give a clear pale yellow filtrate.

Isolation of Isotopic Components

Ammonia—An aliquot of the trichloroacetic acid filtrate was removed. The ammonia was recovered by the addition of 40 per cent alkali with subsequent distillation into 0.07 N sulfuric acid. The remainder of the trichloroacetic acid filtrate was acidified by the addition of 50 ml. of concentrated hydrochloric acid and was taken to dryness *in vacuo*. After repeated distillation with water under reduced pressure, the residue was dissolved in 150 ml. of water and extracted with alcohol-free ether several times. The aqueous layer was again evaporated to dryness *in vacuo*, and the residue was taken up in 300 ml. of alcohol. The inorganic salts were filtered and then washed twice with absolute alcohol until colorless, and the alcoholic solution of amino acid hydrochlorides was concentrated to a syrup. To this syrup were then added 200 ml. of 6 N hydrochloric acid, and the solution was refluxed for 1 hour to hydrolyze any amino acid esters that may have been formed during the alcohol extraction. The excess hydrochloric acid was then removed by repeated distillation with water *in vacuo*.

The various amino acids from the enzymatic experiments were isolated in the usual manner. Glutamic acid was obtained by the procedure of Jones and Moeller (22). All the samples of glutamic acid hydrochloride which were analyzed for N^{15} content were recrystallized to the correct specific rotation, $[\alpha]_D^{20}$ +30.3° to +31.8° (based upon the free acid). The transamination experiments yielded amounts of glutamic acid hydrochloride varying from 75 to 350 mg. Aspartic acid was isolated and recrystallized as the copper salt. Alanine was obtained from the dicarboxylic acid filtrate either as the benzoyl derivative (m.p. 136–138°, $[\alpha]_D^{21}$ +31.7° (1.3 per cent in 1 N NaOH)) or as the β-naphthalenesulfonyl (m.p. 57°, neutral equivalent 296) compound. Leucine and valine were isolated by isoelectric precipitation. Leucine was purified by means of the 2-bromotoluene-

5-sulfonic acid salt, while valine was transformed to the p-toluenesulfonyl derivative, m.p. 148–149° (23). Glycine was isolated via the trioxalato-chromiate complex (24) and purified as the p-toluenesulfonyl compound, m.p. 147–148°. Lysine hydrochloride was prepared from the precipitate formed by the addition of phosphotungstic acid to the dicarboxylic acid filtrate, while histidine was obtained as the dihydrochloride after suitable treatment of the isolated mercury complex.

Results

The experimental demonstration of transamination was based upon the following procedure: an amino acid labeled with N^{15}, α-ketoglutaric acid, and non-isotopic ammonium chloride were added to fresh pig heart homogenate in $KHCO_3$ solution mixture as described above. If the formed glutamic acid contained a higher N^{15} concentration than did the ammonia, then transamination occurred.

It can be seen from Table I that the N^{15} concentration of the isolated glutamic acid was, with two exceptions, higher than in the ammonia. Histidine (Experiment T/37) and ammonia itself (Experiment T/27) are thus excluded as participants in transamination reactions, while these data indicate that transamination did occur between alanine, aspartic acid, leucine, valine, tyrosine, phenylalanine, serine, and to a lesser extent glycine, lysine, and α-ketoglutaric acid. In Experiments T/37 and T/38 a slightly different approach was utilized. Here, the non-isotopic amino acid under investigation was added to the homogenized tissue together with N^{15}-glutamic acid and α-ketoglutarate and was subsequently reisolated from the incubation mixture. An N^{15} concentration higher than ammonia indicates that the amino acid took part in reversible transamination reactions. The known amino acid donor, leucine, was found to contain an appreciable isotope concentration, whereas histidine turned out to be inactive when examined in this fashion. This particular amino acid may not be formed, therefore, by the usual route of amination of its α-keto analogue.

If pyridoxamine is capable of entering into transamination, then the addition of non-isotopic pyridoxamine to a transaminating system containing an isotopic amino acid and α-ketoglutarate should result in a dilution of the N^{15} concentration of the resulting glutamic acid. The aspartic acid-α-ketoglutaric acid pair was chosen for this series of investigations with L-aspartic acid as the isotopic substrate.

The data in Table II show that the N^{15} concentration of the isolated glutamic acid in those experiments wherein normal pyridoxamine was added is about 12 per cent lower than that from the control experiment. The N^{15} concentration of the isolated glutamic acid of Experiment T/17,

in which an equivalent amount of ammonia had been added, was the same as that of the control. These values again indicate that ammonia is not an intermediate in the transamination process. It follows that the dilu-

TABLE I

Representative Amino Acid Transamination in Pig Heart System in Vitro

10 mmoles each of isotopic amino acid, α-ketoglutaric acid, and NH$_4$Cl added to 60 gm. of pig heart homogenate. pH adjusted to 7.4 to 7.5 with solid KHCO$_3$ when necessary. Incubation at 37° for 1 hour.

Experiment No.	Isotopic amino acid added		N^{15} concentration in atom per cent excess of compounds isolated			
	Compound	N^{15} atom per cent excess	Amino group donor	Glutamic acid	Ammonia
T/11	L-Alanine	1.97	1.71	1.54	0.050
T/13*	"	1.97	1.68	1.43	0.054
T/17†	L-Aspartic acid	4.06	3.55	3.13	0.045
T/27‡	NH$_4$Cl	2.92		0.081	2.40
T/5	L-Leucine	1.51	1.37	0.417	0.010
T/23§	DL-Valine	2.32	2.22	1.64	0.066
T/36‖	L-Phenylalanine	1.94		0.287	0.017
T/31	DL-Tyrosine	1.01	0.95	0.108	0.045
T/24	L-Serine	0.602		0.071	0.040
T/20	Glycine	2.17	2.17	0.123	0.053
T/40¶	L-Lysine	4.40	4.44	0.226	0.068
T/38**	L-Glutamic acid + normal L-leucine	1.47	0.321		0.045
T/37††	"　　　" + " L-histidine	1.47	0.018		0.041

* Incubation mixture 0.02 M with respect to arsenite.

† 5 mmoles of NH$_4$Cl present instead of usual amount.

‡ 10 mmoles of normal L-aspartic acid added as amino donor.

§ Tosylvaline isolated predominantly D isomer, $[\alpha]_D^{23}$ −20.4° (1 per cent in ethanol). Compare this rotation with that of Hinman *et al.* (23).

‖ 4 mmoles of each constituent initially added.

¶ Isotope only in α-amino group.

** 5.0 mmoles of each substrate added; 0.02 M arsenite present; leucine reisolated.

†† 5.25 mmoles of each substrate added; 0.02 M arsenite present; histidine reisolated.

tion caused by the addition of pyridoxamine to the reaction medium was not due to deamination of this compound with concomitant reductive amination of α-ketoglutarate.

The actual transfer of the amino group from pyridoxamine to α-ketoglutaric acid was demonstrated by the incubation of N^{15}-pyridoxamine in the homogenate under various conditions (protocols given in Table III). Transamination was the principal reaction undergone by this compound,

although a limited amount of deamination took place as well. In Experiment T/30, both normal aspartic acid and isotopic pyridoxamine were added to the incubation mixture with α-ketoglutarate. Here the N^{15} concentration of the resulting glutamic acid was much lower than that in the

TABLE II

Effect of Pyridoxamine Addition on Aspartic Acid-Glutamic Acid Transamination

10 mmoles of L-aspartic acid, N^{15} 4.06 atom per cent excess, and 10 mmoles of α-ketoglutarate in the pig heart system; pH 7.4 to 7.5. Incubation at 37° for 1 hour. Other additions as indicated.

Experiment No.	Compounds added	N^{15} concentration in atom per cent excess of compounds isolated		
		Aspartic acid	Glutamic acid	NH_3
T/25	None	3.61	3.15	0.199
T/17	5 mmoles NH_4Cl	3.55	3.13	0.045
T/19	5 " pyridoxamine	3.50	2.73	0.151
T/18	5 " " + 5 mmoles ATP	3.46	2.83	0.115
T/26	10 mmoles pyridoxamine	3.52	2.77	0.258

TABLE III

Enzymatic Transamination of Pyridoxamine with α-Ketoglutaric Acid

6.2 mmoles of N^{15}-pyridoxamine and 6.2 mmoles of α-ketoglutaric acid in 60 gm. of pig heart homogenate. Other non-isotopic additions as indicated. Incubation at 37° for 1 hour and at pH 7.4 to 7.5.

Experiment No.	Constituents added	N^{15} atom per cent excess	N^{15} concentration in atom per cent excess of compounds isolated		
			Aspartic acid	Glutamic acid	NH_3
T/29	Pyridoxamine	1.42		0.506	0.105
T/30	" + 6.2 mmoles aspartic acid	1.42	0.089	0.071	0.113
T/32	" + 6.2 " NH_4Cl	1.31		0.363	0.024
T/33	" + 6.2 " deoxypyridoxine	1.31		0.664	0.102

other experiments, owing to the competitive transamination of the aspartic acid. However, the aspartic acid and glutamic acid samples isolated at the end of the reaction contained almost the same N^{15} concentration. The higher N^{15} concentration of the ammonia sample in this experiment is probably due to deamination of the isotopic pyridoxamine in some manner. This result again demonstrates the non-equilibration of the ammonia pool with the transamination process.

An analogue of pyridoxine, 2,4-dimethyl-3-hydroxy-5-hydroxymethyl-pyridine, or deoxypyridoxine, has been shown by Ott to act as a powerful antagonist of pyridoxine in the chick (25). A similar effect upon rats was demonstrated by Emerson (26). On the other hand, this compound had no apparent effect upon an aspartic-glutamic transaminase from heart muscle, although it could be shown that deoxypyridoxine phosphate inhibited a tyrosine decarboxylase preparation (27). In Experiment T/33, addition of an equimolar amount of deoxypyridoxine to the reaction medium in no way inhibited pyridoxamine transamination.

TABLE IV

Comparison of Isotopic Substrates As Amino Donors to α-Ketoglutarate in Pig Heart System

$$E_t = \frac{N^{15} \text{ atom per cent excess isolated glutamic acid}}{N^{15} \text{ atom per cent excess isotopic amino donor}} \times 100$$

Experiment No.	Isotopic constituent added	E_t
T/17	L-Aspartic acid	77
T/6, T/11, T/13	L-Alanine	75 (Average)
T/23	DL-Valine	70
T/4, T/5	L-Leucine	28 (Average)
T/32	Pyridoxamine	28
T/36	L-Phenylalanine	15
T/24	L-Serine	12
T/31	DL-Tyrosine	11
T/20	Glycine	5.7
T/40	L-Lysine	5.1
T/27	NH₄Cl	2.8

In Table IV a rough comparison between the activities of the amino acids tested, pyridoxamine, and ammonia as donors of the amino group to α-ketoglutarate is given. Aspartic acid, alanine, and valine were the most active, followed by leucine, pyridoxamine, phenylalanine, serine, and tyrosine. Lysine and glycine were poor participants, while histidine, discussed previously, was essentially inactive and not listed. It is important to recognize, however, that the data presented in Tables I to IV are by no means a quantitative measure of the extent of transamination. In order to determine the quantitative relationships between the transaminase enzymes their concentrations in the tissue, as well as their equilibrium constants, must be estimated.

That these additional transaminations were enzymatic in nature can be deduced from the fact that the L isomer of glutamic acid was isolated in all instances. Furthermore, leucine and α-ketoglutarate in one case, and

pyridoxamine and α-ketoglutarate in another, were incubated for an hour at 37° in $KHCO_3$ solution of pH 7.4 in the same concentrations as those in the enzymatic runs. Samples were withdrawn at this point, and also after acidification with HCl, and were examined by paper partition chromatography for the presence of glutamic acid. There was no evidence for non-enzymatic transamination.

<div align="center">DISCUSSION</div>

Presumptive evidence for the enzymatic transaminations between pyridoxamine and α-keto acids has been reported previously only in bacterial systems. In experiments with *Streptococcus faccalis* Bellamy *et al.* (28) showed that pyridoxamine was transformed into pyridoxal phosphate upon the addition of pyruvate to the medium. Similarly, the interconversion of pyridoxamine phosphate to pyridoxal phosphate with pyruvate, and presumably other α-keto acids, catalyzed by enzymes from *Clostridium welchii* has been studied (29). The demonstration here that pyridoxamine-α-ketoglutarate transamination occurs in pig heart tissue is not unexpected in the light of the recent developments in comparative biochemistry. These findings, together with the recent disclosure that purified aspartic-glutamic apotransaminase can be activated by either pyridoxal or pyridoxamine phosphate (30), strengthen the original working hypothesis put forth by Snell for the mechanism of transamination.

The transamination of pyridoxamine with α-ketoglutarate can be envisioned as involving either Mechanism 1 or 2.

(1) $PyN^{15}H_2 + ATP \xrightleftharpoons{\text{kinase}} PyN^{15}H_2 \cdot PO_4 + \text{adenosine diphosphate}$

$PyN^{15}H_2 \cdot PO_4 + \text{enzyme} \rightleftharpoons \text{enzyme} \cdot Py \cdot N^{15}H_2 \cdot PO_4$

(2) $\text{Enzyme} \cdot Py \cdot PO_4 \cdot \text{aldehyde} + Py'N^{15}H_2 \rightleftharpoons$

$\text{enzyme} \cdot Py \cdot PO_4 \cdot N^{15}H_2 + Py' \cdot \text{aldehyde}$

followed by

(3) $\text{Enzyme} \cdot Py \cdot PO_4 \cdot N^{15}H_2 + \alpha\text{-ketoglutarate} \rightleftharpoons$

$N^{15}\text{-glutamate} + \text{enzyme} \cdot Py \cdot PO_4 \cdot \text{aldehyde}$
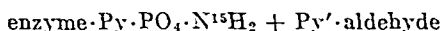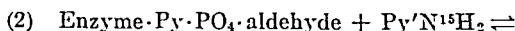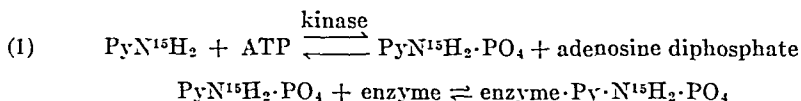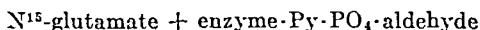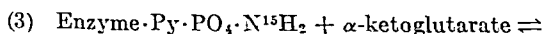
Whether pyridoxamine *per se* or its phosphorylated derivative entered into the transamination in this pig heart system cannot be inferred from these experiments. Pyridoxamine kinase has been demonstrated in yeast (31) and the presence of the aldehyde kinase indicated in brain (32). Certainly, if phosphorylation is involved prior to the entrance of the amine into transamination in the pig heart homogenate, all the necessary components for pyridoxamine kinase reactions must be present in

this tissue. It should be noted that the addition of ATP to the system (Experiment T/18, Table II) did not enhance isotope incorporation into glutamate. On the other hand, it must not be overlooked that perhaps the phosphorylated coenzyme, already on the enzyme surface, is handling the N^{15}-labeled pyridoxamine as it would any other amino donor. In this case, a transamination between the amine and aldehyde forms of vitamin B_6, with the molecule acting both as a substrate and as the coenzyme, must be postulated. Meister and coworkers report, however, that they have been unable to demonstrate the conversion of enzyme-pyridoxamine phosphate to enzyme-pyridoxal phosphate (30).

I should like to thank Dr. David Shemin for his advice and encouragement during this investigation.

SUMMARY

1. An examination of the transamination reaction in a pig heart homogenate with N^{15}-labeled substrates has been undertaken. An experimental method has been developed whereby it is possible to determine whether an amino donor undergoes transamination to α-ketoglutarate.

2. Besides alanine and aspartic acid, leucine, valine, phenylalanine, tyrosine, serine, glycine, and lysine are capable of entering such transamination. Histidine appears to be inert as an α-amino donor. Ammonia is not an intermediate in the amino group transfer.

3. Pyridoxamine labeled with N^{15} in the aminomethyl group has been synthesized. It transaminates readily with α-ketoglutarate in this system. The relationship of these findings to the mechanism of transamination has been discussed.

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THE PREPARATION OF SEDOHEPTULOSE DIPHOSPHATE

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Sedoheptulose diphosphate was discovered in the course of efforts to isolate D-erythrose 4-phosphate formed from sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate in the reaction catalyzed by transaldolase (1). In the presence of crystalline muscle aldolase and fructose diphosphate used as the source of glyceraldehyde 3-phosphate, the tetrose ester was found to condense with dihydroxyacetone phosphate to form a new heptulose diphosphate ester. This product was purified by ion exchange chromatography and identified as sedoheptulose 1,7-diphosphate.

(1) Fructose diphosphate $\xrightleftharpoons{\text{aldolase}}$ glyceraldehyde 3-phosphate
                                    + dihydroxyacetone phosphate

(2) Sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate $\xrightleftharpoons{\text{transaldolase}}$
                                    erythrose 4-phosphate + fructose 6-phosphate

(3) Erythrose 4-phosphate + dihydroxyacetone phosphate $\xrightleftharpoons{\text{aldolase}}$
                                    sedoheptulose 1,7-diphosphate

The preparation of sedoheptulose diphosphate by this series of reactions (2) involves the prior isolation of sedoheptulose 7-phosphate and is unsuitable for large scale operations. An alternative method for the preparation of this compound was suggested by the work of Racker *et al.* (3, 4), who have described the formation of heptulose phosphate from fructose 6-phosphate and ribose 5-phosphate in the presence of transketolase. Upon addition of fructose diphosphate and aldolase, heptulose formation was substantially increased. It was suggested by these workers that this increase was due to the formation of sedoheptulose diphosphate, although this product was not isolated or identified.

In the present paper the isolation of sedoheptulose diphosphate from a similar reaction mixture, containing only fructose diphosphate and fruc-

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tose 6-phosphate as substrates, is described. These reactions provide presumptive evidence for erythrose 4-phosphate formation in the transketolase-catalyzed cleavage of fructose 6-phosphate. In the presence of transketolase "active glycolaldehyde"[1] condenses with glyceraldehyde 3-phosphate to form pentose phosphate, which reacts further to produce sedoheptulose 7-phosphate.

$$
\begin{array}{l}
\text{Fructose 6-} \quad \xrightleftharpoons{\text{transketolase}} \quad
\left\{
\begin{array}{c}
\text{"active glycolaldehyde"} \\
+ \\
\text{erythrose 4-phosphate}
\end{array}
\right\} \\[2em]
\text{Fructose di-} \quad \xrightleftharpoons{\text{aldolase}} \quad
\left\{
\begin{array}{c}
\text{dihydroxyacetone phos-} \\
\text{phate} \\
+ \\
\text{glyceraldehyde 3-phosphate}
\end{array}
\right\}
\end{array}
\xrightleftharpoons{\text{aldolase}}
\begin{array}{l}
\text{sedoheptu-} \\
\text{lose di-} \\
\text{phos-} \\
\text{phate}
\end{array}
$$
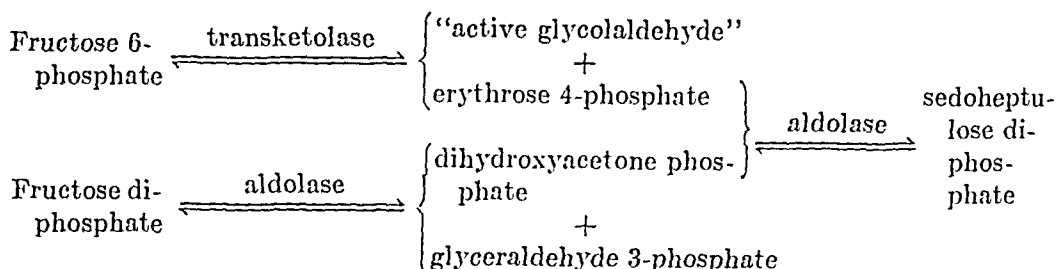
Although the over-all yield of sedoheptulose diphosphate is only 30 per cent, even with a 3-fold excess of fructose diphosphate, the starting compounds are commercially available, and the product is readily separated from the other reaction products, including sedoheptulose 7-phosphate, by ion exchange chromatography. By an exchange reaction with appropriately labeled fructose diphosphate in the presence of aldolase, C^{14} can be introduced into any of the first three positions of the heptulose ester.

Materials

Commercial barium and magnesium salts of fructose 6-phosphate and fructose diphosphate were dissolved in dilute hydrochloric acid and converted to the potassium salts by passage through a Dowex 50-K$^+$ column. Uniformly labeled fructose diphosphate was obtained from the Nuclear Instrument and Chemical Corporation. For chromatography, Dowex 1 anion exchange resin (The Dow Chemical Company, 10 per cent crosslinked, 200 to 400 mesh) was washed with 2 N hydrochloric acid until the washings were free of significant absorption at 260 mμ. It was converted to the formate form by treatment with 2 M sodium formate until the chloride was completely displaced. Excess formate was removed by washing with water. Reduced diphosphopyridine nucleotide was prepared by the method of Ohlmeyer (6) from diphosphopyridine nucleotide obtained by the method of Kornberg and Pricer (7). Triphosphopyridine nucleotide (TPN) was prepared according to Kornberg and Horecker (8).

L-Xylulose was kindly furnished by Dr. O. Touster of Vanderbilt University. D-Ribulose was prepared from D-arabinose by the method of Glatthaar and Reichstein (9) and D-xylulose by the oxidation of D-arabitol

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[1] "Active glycolaldehyde" designates the presumed 2-carbon intermediate formed in the transketolase reaction (5).

by *Acetobacter suboxydans* (10). L-Erythrulose was prepared from erythritol with *A. suboxydans* according to Müller, Montigel, and Reichstein (11).

Spinach transketolase was prepared as previously described (12). Ammonium Sulfate III fraction, dialyzed overnight against 0.1 M sodium acetate of pH 7.2, was used. *Zwischenferment* was prepared by the method of Kornberg (13). Aldolase, nine times recrystallized, was prepared according to the method of Taylor *et al.* (14). Hexosephosphate isomerase was a crude rabbit muscle fraction obtained as a by-product of the aldolase preparation. The fraction precipitated by ammonium sulfate between 20° and 50 per cent saturation was stored as a paste at 5° and dissolved as needed. Glycerophosphate dehydrogenase was a crude preparation from rabbit muscle (15).

Analytical Methods

Spectrophotometric measurements were made at room temperature with a Beckman model DU spectrophotometer in 1.0 cm. cells. Radioactivity measurements were made at infinite thinness with a windowless gas flow counter.

Sedoheptulose diphosphate was determined by the orcinol reaction (16) as previously described (12). However, since the presence of high concentrations of fructose esters interfered with the determinations, this method could not be used to follow accurately the formation of sedoheptulose diphosphate in the incubation mixture, and it was necessary to carry out a chromatographic separation before reliable determinations of heptulose could be made.

Inorganic phosphate was determined by the method of Fiske and Subbarow (17). Organic phosphate was converted to inorganic phosphate by a wet ashing procedure.

Fructose 6-phosphate was determined spectrophotometrically with TPN, *Zwischenferment*, and hexosephosphate isomerase (12). Fructose diphosphate was also determined spectrophotometrically with reduced diphosphopyridine nucleotide and glycerophosphate dehydrogenase (15).

Preparation of Sedoheptulose Diphosphate

Incubation—A mixture of 1850 μmoles of potassium fructose 6-phosphate and 4440 μmoles of potassium fructose diphosphate was adjusted to pH 7.0 with 2.0 N potassium hydroxide. No further buffer was needed. The reaction was begun by the addition of 61.0 mg. of spinach transketolase and 118.0 mg. of rabbit muscle aldolase. The final volume was made up to 1.0 liter. For a qualitative measure of the progress of the reaction, samples were taken for orcinol assay (with a 10 minute heating period)

and the 580 mμ/670 mμ density ratio computed. When this ratio reached a constant level, the reaction was assumed to have reached equilibrium. In this experiment 90 minutes at room temperature were required.

Chromatography—The entire incubation mixture (1 liter), without deproteinization, was placed on a Dowex 1-formate column, 8.2 sq. cm. $\times$ 38 cm., and washed with 200 ml. of water. The diphosphate ester was eluted with a gradient elution technique (18), with a mixing bottle containing 2.0 liters of water, and 0.2 M formic acid containing 0.5 M sodium formate in the reservoir. Fractions of 50 ml. were collected at the rate of 3.0 ml. per minute and analyzed for heptulose. Sedoheptulose 7-phosphate appeared after 1.5 liters of effluent had passed through the column; sedoheptulose diphosphate after 4.0 liters of effluent.

Precipitation of Barium Salt—The fractions in the sedoheptulose diphosphate peak were pooled and adjusted to approximately pH 5.0 with saturated sodium hydroxide. 3.3 ml. of 1 M barium acetate were added, and the pH was adjusted to 6.3 with saturated barium hydroxide, following which 1 volume of absolute ethanol was added. After 30 minutes at 0° the precipitate was collected by centrifugation, washed once with 50 ml. of 80 per cent ethanol, and dried *in vacuo* over KOH. The dried product (585 mg.) contained 72 per cent by weight of barium sedoheptulose diphosphate (660 μmoles) and had less than 2 per cent of hexose diphosphate as an impurity. It contained no inorganic phosphate, and the ratio of total phosphate to sedoheptulose was 1.96.

Preparation of Sedoheptulose Diphosphate-1,2,3-C^{14}

Both sedoheptulose diphosphate and fructose diphosphate are rapidly cleaved by crystalline muscle aldolase; in each case one of the products is dihydroxyacetone phosphate. It is therefore convenient to introduce C^{14} into positions 1, 2, and 3 of the sedoheptulose diphosphate molecule by incubating it with aldolase in the presence of radioactive fructose diphosphate. The following experiment with the uniformly labeled compound illustrates this method.

Equilibration—A solution containing 423 μmoles of potassium sedoheptulose diphosphate and 55.0 μmoles of potassium fructose diphosphate, uniformly labeled (8.1 $\times$ 10^6 c.p.m.[2]) in a total volume of 43 ml., was adjusted to pH 7.0 with 2 N potassium hydroxide. The solution was incubated at room temperature for 90 minutes with 7.85 mg. of recrystallized rabbit muscle aldolase.

Chromatography—After 90 minutes the reaction mixture was chromato-

[2] It has not been established that all of the radioactivity in the commercial product was associated with fructose diphosphate. Following incubation and chromatography, only about 60 per cent of the total counts was recovered.

graphed as described previously with a 7.1 sq. cm. × 15.3 cm. Dowex 1-formate column and a mixing bottle containing 1200 ml. of water. 15 ml. fractions were collected at the rate of 1 ml. per minute. Radioactive sedoheptulose diphosphate appeared after 1.2 liters as determined by the orcinol reaction (Fig. 1). The sedoheptulose diphosphate peak was closely followed by a second radioactive peak containing fructose diphosphate.

Precipitation of Sedoheptulose Diphosphate—Fractions 87 to 104 were pooled and adjusted to approximately pH 5.0 with 1.0 ml. of saturated sodium hydroxide. 4.5 ml. of 1.0 M barium acetate were added. The pH
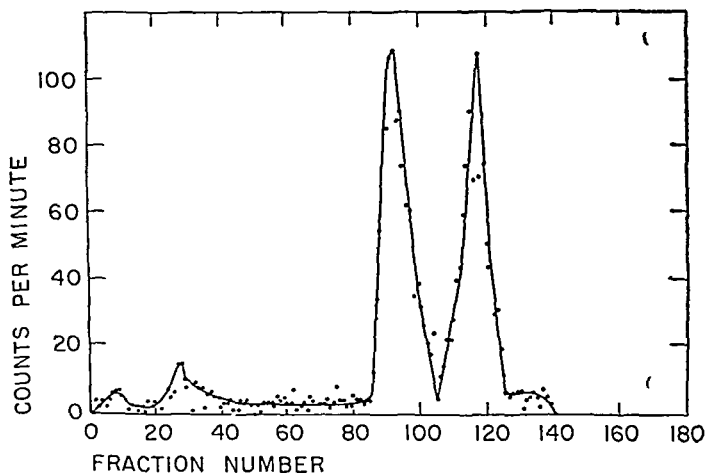
Fig. 1. Separation of sedoheptulose diphosphate and fructose diphosphate by ion exchange chromatography. Radioactivity measurements were made with a 0.05 ml. aliquot. The first major peak was found to contain sedoheptulose diphosphate as determined by the orcinol reaction and the second large peak fructose diphosphate as determined by enzymatic assay.

was then adjusted to 6.5 with 1.0 ml. of saturated barium hydroxide, and 1 volume of absolute ethanol was added. The suspension was kept at 0° for 30 minutes, centrifuged, and the precipitate was washed with 25.0 ml. of 80 per cent ethanol and dried *in vacuo.* The yield was 291.0 mg., containing 400 μmoles of sedoheptulose diphosphate (87 per cent pure). The specific activity was 4.0×10^3 c.p.m. per μmole.

Precipitation of Fructose Diphosphate—Fractions 110 to 123 were pooled and adjusted to approximately pH 5.0 with 0.7 ml. of saturated sodium hydroxide and treated with 4.5 ml. of 1.0 M barium acetate. The pH was then adjusted to 6 3 with 1.8 ml. of saturated barium hydroxide, and 1 volume of absolute ethanol was added. The suspension was kept at 0° for 30 minutes, centrifuged, and the precipitate was washed with 25.0 ml. of 80 per cent ethanol and dried *in vacuo.* The yield was 57.7 mg.

containing 40 μmoles of barium fructose diphosphate with a specific activity of 4.0×10^4 c.p.m. per μmole.

Transketolase Activity with Erythrose 4-Phosphate

The transketolase assay method employed in previous work (12) depends on the formation of triose phosphate from pentose phosphate. It is therefore unsuitable for use with compounds which, on cleavage, would not yield triose phosphate. The preparation of sedoheptulose diphosphate

FIG. 2. Ability of transketolase to form "active glycolaldehyde" from the non-phosphorylated sugars. The assay system contained 1.0 μmole of potassium sedoheptulose diphosphate, 34 μmoles of triethanolamine buffer of pH 7.5, 9 μmoles of ethylenediaminetetraacetate, 0.7 μmole of TPN, 0.08 mg. of aldolase, 0.06 mg. of spinach transketolase (0.72 unit, Ammonium Sulfate IV (12)), 2.6 mg. of hexosephosphate isomerase, and 7.0 mg. of *Zwischenferment*. The sugars were tested at a level of 0.4 μmole. The total volume in the cell was 1.04 ml.

has made available an alternative assay for this enzyme based on the ability of a substrate to form "active glycolaldehyde."

$$\text{Sedoheptulose diphosphate} \xrightleftharpoons{\text{aldolase}} \text{erythrose 4-phosphate}$$
$$+ \text{ dihydroxyacetone phosphate}$$

$$\text{"Active glycolaldehyde"} + \text{erythrose 4-phosphate} \xrightleftharpoons{\text{transketolase}}$$
$$\text{fructose 6-phosphate}$$

The rate of fructose 6-phosphate formation was followed with an excess of the hexosephosphate isomerase-*Zwischenferment*-TPN system (12) (Fig. 2). In this assay system, ribulose 5-phosphate showed maximal activity, while L-erythrulose was nearly as active. No activity was detected with

D-xylulose, L-xylulose, or D-ribulose. When the level of these sugars was increased 10-fold, to 4.0 μmoles, there still was no perceptible activity.

DISCUSSION

It has been suggested on the basis of isotope studies (2) that erythrose 4-phosphate condenses with "active glycolaldehyde" to form hexose monophosphate. More direct evidence for such a reaction catalyzed by transketolase is provided by the observation that fructose 6-phosphate is cleaved by this enzyme (3). The formation of erythrose 4-phosphate as a product of this cleavage is now confirmed by the isolation of sedoheptulose 1,7-diphosphate. Direct evidence for the reversal of this reaction is derived from the series of reactions catalyzed by aldolase and transketolase, in which sedoheptulose diphosphate gives rise to fructose 6-phosphate in the presence of "active glycolaldehyde" donors such as ribulose 5-phosphate or erythrulose.

The inability of the non-phosphorylated sugars, ribulose and xylulose, to serve as "active glycolaldehyde" donors is rather unexpected, since it has been demonstrated (5, 12) that glyceraldehyde will serve as an "active glycolaldehyde" acceptor. Apparently the condensations involving the non-phosphorylated aldotrioses are difficult to reverse. A similar observation has been made in the case of the condensations catalyzed by aldolase (19).

In the exchange of isotope between fructose diphosphate and sedoheptulose diphosphate, the data are consistent with the complete equilibration of the dihydroxyacetone phosphate moieties. The isotope present in carbon atoms 4, 5, and 6 of fructose diphosphate (50 per cent of the total radioactivity) would not be expected to exchange. On the assumption of complete equilibration, the other half of the fructose diphosphate molecule (55 μmoles) would be diluted 9-fold by dihydroxyacetone phosphate contributed by sedoheptulose diphosphate (423 μmoles), leaving 56 per cent of the total activity in hexose diphosphate, with 44 per cent in the heptulose ester. Of the radioactivity recovered from the chromatogram, 55 per cent was accounted for as fructose diphosphate and 45 per cent as sedoheptulose diphosphate, when suitable corrections are made for losses during chromatography.

The suggestion (2) that sedoheptulose diphosphate may function as a source of the tetrose ester has received support from the work of Srinivasan *et al.* (20), in which evidence was obtained for the participation of both halves of the molecule in the enzymatic synthesis of shikimic acid and hence of the benzene ring of the aromatic amino acids.

Erythrose 4-phosphate has now been synthesized by Ballou, Fischer, and McDonald (21), who have confirmed the aldolase-catalyzed con-

densation leading to the sedoheptulose diphosphate. These compounds have already been shown to participate in a number of biological processes (22). Their availability by synthetic and enzymatic procedures should facilitate further studies.

SUMMARY

A new enzymatic synthesis of sedoheptulose diphosphate is described, with fructose 6-phosphate and fructose diphosphate as substrates.

By an exchange reaction catalyzed by aldolase, radioactivity can be transferred from suitably labeled fructose diphosphate to any of the first 3 carbon atoms of sedoheptulose diphosphate.

Under conditions which were suitable for the cleavage of erythrulose and ribulose 5-phosphate by transketolase, the non-phosphorylated sugars ribulose and xylulose were not attacked.

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THE RÔLE OF XYLULOSE 5-PHOSPHATE IN XYLOSE METABOLISM OF LACTOBACILLUS PENTOSUS

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Lampen (1) observed that cell-free extracts of *Lactobacillus pentosus* grown on D-xylose catalyzed the disappearance of D-xylose in the presence of ATP.[1] The phosphate esters which accumulated were identified as a mixture of D-ribose 5-phosphate and D-ribulose 5-phosphate. Neither D-xylose 5-phosphate nor D-xylulose 5-phosphate was found in the reaction mixture. Mitsuhashi and Lampen (2) also demonstrated the presence of xylose isomerase in these extracts and, on the basis of the fact that D-xylulose was esterified more rapidly than D-xylose, it was postulated that the following reaction sequence takes place.

$$\text{D-Xylose} \rightleftharpoons \text{D-xylulose} \xrightarrow{\text{ATP}} \text{xylulose 5-phosphate} \rightleftharpoons$$

$$\text{ribulose 5-phosphate} \rightleftharpoons \text{ribose 5-phosphate}$$
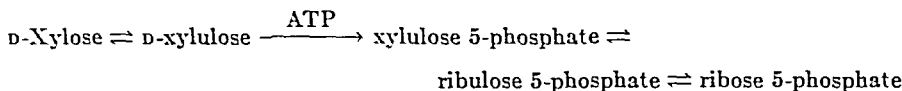
Hochster (3) has obtained evidence for a similar series of reactions in *Pseudomonas hydrophila*. With extracts of this organism D-xylose is converted to a mixture of pentose phosphates, including a phosphate ester of xylulose. Slein (4) has obtained extracts of *Pasteurella pestis* which contain a xylose isomerase and a xylulose kinase.

It is clear from these results (1–4) that a mechanism exists for the conversion of xylulose 5-phosphate to ribulose 5-phosphate. Since transketolase cleaves substrates with either configuration with respect to the hydroxyl group at carbon atom 3 (5), this enzyme might be expected to provide such a mechanism. Thus a cleavage of xylulose 5-phosphate to "active glycolaldehyde" and triose phosphate, followed by a recondensation of these fragments, would be expected to produce both epimeric ketose esters. However, Ashwell and Hickman (6) have described an enzyme

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* Postdoctoral Fellow of the United States Public Health Service, 1954–55.

[1] The following abbreviations are used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPN+, oxidized diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane; Ru-5-P, D-ribulose 5-phosphate; Xu-5-P, D-xylulose 5-phosphate; PKPE, phosphoketopentoepimerase; ATPase, adenosine triphosphatase; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid; TK, transketolase.

preparation from mouse spleen which converts ribose 5-phosphate to a mixture of ribulose phosphate and xylulose phosphate in the absence of transketolase. They suggest the existence of a specific enzyme which catalyzes the equilibration between the two pentose phosphates.

In the present communication data are presented for the occurrence, in cell-free extracts of *L. pentosus* grown on xylose, of both a specific xylulose kinase and a specific epimerase. The latter enzyme catalyzes the equilibration of D-xylulose 5-phosphate and D-ribulose 5-phosphate and will be referred to as phosphoketopentoepimerase (PKPE). The isolation and properties of D-xylulose 5-phosphate are also recorded. This ester is cleaved by purified spinach transketolase at the same rate as is ribulose 5-phosphate. It is thus established that spinach transketolase has no specificity for a particular configuration of the hydroxyl group of the 3rd carbon atom of the ketopentose.[2]

Methods

Materials—Barium ribulose 5-phosphate was prepared by enzymatic oxidation of 6-phosphogluconate by yeast 6-phosphogluconic dehydrogenase (7). DPNH was prepared by the method of Ohlmeyer (8). D-Xylose (anhydrous) was obtained from Professor W. Z. Hassid of the University of California. Lithium phosphoenolpyruvate (91 per cent pure) was supplied by Mr. William E. Pricer of this Institute. D-Xylulose was prepared by the oxidation of D-arabitol by *Acetobacter suboxydans* (9). Paper chromatographic analysis of the preparation showed it to be free of D-ribulose and D-xylose. Other substrates and cofactors were commercial preparations.

Glycerophosphate dehydrogenase and hexosephosphate isomerase were prepared from rabbit muscle extract by precipitation with ammonium sulfate (10). The fraction which precipitated between 0.2 and 0.5 saturation with ammonium sulfate was used as a source of hexosephosphate isomerase, and the fraction collected between 0.5 and 0.7 saturation was the source of glycerophosphate dehydrogenase. Crystalline aldolase was prepared according to the method of Taylor *et al.* (11). Acid phosphatase was purified from potato by the method of Kornberg.[3] Pyruvate kinase and

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[2] Recently, highly purified preparations of spinach transketolase have been obtained which show little activity with ribulose 5-phosphate unless a phosphoketo pentoepimerase preparation is added. The apparent lack of specificity of the spinach transketolase preparations employed in the present work may be due to the presence of phosphoketopentoepimerase in these preparations. Srere and Racker (personal communication) have now shown that yeast transketolase is specific for xylulose 5-phosphate and other substrates with the trans configuration. The same may be true for the spinach enzyme.

[3] Unpublished procedures.

lactic dehydrogenase were isolated by the method of Kornberg and Pricer (12). We are indebted to Dr. J. Hurwitz of this Institute for a generous supply of spinach phosphoribulokinase (13).

Dowex 1, 10 per cent cross-linked, was used in the formate form.

Analytical Methods—Spectrophotometric measurements were made at room temperature with a Beckman model DU spectrophotometer. Pentoses were measured by the orcinol test according to Mejbaum (14), except that heating was continued for 40 minutes.

The per cent of xylulose in a mixture of ribulose and xylulose was determined by the carbazole method (15) as modified by Ashwell and Hickman (6). The method is based on the fact that color development with ribulose in the cysteine-carbazole reaction is more rapid than with xylulose. Two readings are taken at 540 mμ in the Coleman junior spectrophotometer, the first at 15 minutes and the second at 120 minutes. The per cent of xylulose in a mixture may then be calculated by the increase in absorption in the 15 to 120 minute interval, based on the behavior of authentic standards.

Inorganic phosphate was estimated by the method of Fiske and Subbarow (16). Total organic phosphate was determined by a wet ashing procedure.

ADP was estimated by a modification of the spectrophotometric method of Kornberg and Pricer (12). Protein was determined by the turbidimetric method of Bücher (17).

For the enzymatic hydrolysis of pentose phosphates 8 μmoles of organic phosphate, 0.02 ml. of 0.5 M acetate buffer at pH 5.2, 0.01 ml. of 0.1 M cysteine at pH 6.5, 0.01 ml. of 0.1 M MgCl$_2$, and 0.02 ml. of potato phosphatase (17 units) in a total volume of 0.56 ml. were incubated for 120 minutes at 37°. The inorganic phosphate liberated was removed by addition of 0.02 ml. of 1 M barium acetate and 4 volumes of ethanol. The suspension was centrifuged and the supernatant solution lyophilized. The residue containing the free pentoses was dissolved in 0.5 ml. of water.

Chromatography was carried out on Whatman No. 1 paper with water-saturated phenol by a descending method. The chromatograms were sprayed with TCA-orcinol (18) and heated at 100° for 5 minutes. Sugars were identified by comparison with the R_F values of known pentoses and by the characteristic gray-blue color of xylulose, the red-orange of ribulose, and the fluorescent properties of ribulose when viewed under ultraviolet irradiation.

Enzyme Assays—Two methods were employed for the assay of xylulose kinase. The first, used in preliminary observations, depended on the disappearance of free pentose as assayed by the orcinol reaction after the phosphate esters had been removed as alcohol-insoluble barium salts. The

reaction mixture contained 4 μmoles of ATP, 2 μmoles of D-xylose, 10 μmoles of $MgCl_2$, 20 μmoles of Tris buffer at pH 7.5, and enzyme to a total volume of 0.8 ml. Aliquots of 0.1 ml. were removed at intervals, added to 0.5 ml. of ethanol, and treated with 0.01 ml. of 20 per cent barium acetate. The barium salts were removed by centrifugation, and the supernatant solution was assayed directly for total pentose content. This assay was employed with crude enzyme extracts containing xylulose isomerase, which permitted the use of D-xylose as a substrate.

The second method was based on the appearance of ADP with D-xylulose as the substrate and was employed when xylose isomerase was absent. The spectrophotometric procedure of Kornberg was followed with the inclusion of proper controls for ATPase activity. The assay system consisted of 0.6 μmole of phosphoenolpyruvate, 0.45 μmole of ATP (Sigma, crystalline), 0.5 unit of pyruvate kinase, 2 units of lactic dehydrogenase, 8 μmoles of phosphate buffer at pH 7.5, 1 μmole of magnesium chloride, 1 μmole of cysteine at pH 7.5, and 0.24 μmole of DPNH. The final volume was 1.2 ml. Xylulose kinase and 0.3 μmole of D-xylulose were added rapidly. Xylulose was omitted from one cuvette; measurements of DPNH oxidation in this cuvette reflect ATPase activity. 1 unit was defined as the amount of enzyme required for a decrease of optical density of 1.0 per minute under the conditions of the test, corrected for ATPase activity. ATPase units were similarly defined. Specific activity is represented by the number of units per mg. of protein.

Spinach transketolase was assayed by the method of Horecker *et al.* (5).

Purification of Xylulose Kinase

Preparation of Cell-Free Extracts—*L. pentosus* strain 124-2 (ATCC 8041) was grown on a xylose-enriched medium from a culture provided by Professor H. A. Barker of the University of California. The culture medium contained 0.4 per cent Difco yeast extract, 1 per cent Difco nutrient broth, 1 per cent sodium acetate, 1 per cent D-xylose, 0.1 per cent glucose, 0.02 per cent $MgSO_4 \cdot 7H_2O$, 0.001 per cent NaCl, 0.001 per cent $FeSO_4 \cdot 7H_2O$, and 0.001 per cent $MnSO_4 \cdot 4H_2O$. D-Xylose was autoclaved separately as a 10 per cent solution and then added aseptically to a sterilized medium. Incubations were for 24 hours at 37°. The cells were harvested at 2° with a Sharples supercentrifuge, washed with 0.02 M $NaHCO_3$, and stored at $-16°$ as a thick paste. The frozen paste could be stored indefinitely with little loss in kinase activity. The yield was approximately 1.2 gm. per liter of medium. A suspension of 10 gm. of cells (wet weight) in 20 ml. of 0.01 M $NaHCO_3$ was exposed to a 9 kc. Raytheon sonic oscillator for 1 hour. Cell-free extracts were obtained by centrifuging the treated cells for 1 hour at 13,000 $\times$ g in the International

refrigerated centrifuge. The amber-colored extract (Table I) was stored at 0° for several months with little loss in kinase activity. It contains xylulose isomerase, phosphoriboisomerase, ATPase, xylulose kinase, transketolase, and phosphoketopentoepimerase.

Manganese Step—The cell-free extract was treated with 0.05 volume of 1 M MnCl₂, kept for 30 minutes at 4°, and centrifuged at 13,000 × *g* for 5 minutes. The precipitate containing nucleoprotein and most of the ATPase activity was discarded.

Calcium Phosphate Gel Step—To the clear supernatant solution were added 3 volumes of calcium phosphate gel (19) (17.9 mg., dry weight, per ml.). The suspension was centrifuged at 0° and washed once with 3 volumes of water, after which inactive proteins were eluted with 20 ml. of

TABLE I

Purification of Xylulose Kinase

Step	Total volume	Units per mL.*	Specific activity*	Over-all per cent recovery
	ml			
Crude extract	20	(21)†	(0.24)	
Mn supernatant solution	20	21	0.98	
Calcium phosphate gel eluate	20	16	5.09	80
1st (NH₄)₂SO₄ ppt.	5	27	5.0	32
Heat treatment and (NH₄)₂SO₄ ppt. .	5	21	16.0	25

* As defined in the text.
† Estimated as a minimal value.

0.05 M potassium phosphate buffer, pH 7.7. (Trials should be conducted at this elution step with phosphate buffer solution of varying concentration, since occasionally the kinase is eluted with more dilute buffer.) The first eluate was discarded and the enzyme eluted from the gel with 20 ml. of 0.1 M phosphate buffer at pH 7.7.

First (NH₄)₂SO₄ Step—To the eluate (20 ml.) at 0° were added 30 ml. of saturated (at room temperature) ammonium sulfate solution, and the precipitate was discarded. 4.7 gm. of ammonium sulfate were added to the supernatant solution, and the precipitate was dissolved in water to a final volume of 5 ml.

Acid-Heat Step and Ammonium Sulfate Step—The solution was adjusted to pH 5.4 by the addition of 0.5 M KH₂PO₄. It was then warmed to 53° in 2 minutes and maintained at this temperature for 5 minutes. Higher temperatures produced considerable loss of activity. The heated solution was cooled to 4° and treated with 2.5 volumes of cold saturated ammonium sulfate. The heavy precipitate was discarded, and 1.9 gm. of ammonium

sulfate were added to the supernatant solution. The precipitate was collected, dissolved in 5 ml. of water, and stored at 0°. This preparation contains 21 units of kinase, 1 unit of ATPase, 0.15 unit of transketolase per ml. of enzyme, and PKPE activity.

Properties of Xylulose Kinase—The purified preparations were stored at 0° for several months with little loss in activity. On exposure to 53° for 5 minutes, the following losses were observed: at pH 8, 66 per cent; pH 7.0, 60 per cent; pH 6.2, 50 per cent; pH 5.7, 30 per cent; and pH 5.4, 26 per cent. At 55°, pH 5.4, losses were much greater, and at 60° all activity disappeared.

L-Xylulose, D-ribulose, L-erythrulose, D-xylose, and D-ribose are inert as substrates. Since the enzyme is specific for D-xylulose, it can be employed for the spectrophotometric determination of small concentrations of D-xylulose in the presence of other sugars.

The pH optimum lies on a rather broad plateau between pH 7.0 and 7.9.

The enzyme is activated by a number of divalent cations. At a final concentration of 0.005 M, the following quantities of ADP (in micromoles) were formed in 5 minutes at 25°: Mg^{++}, 0.17; Mn^{++}, 0.16; Fe^{++}, 0.19; and Zn^{++}, 0.17. Without addition of metal 0.08 μmole of ADP was found. The reaction mixture in these experiments contained 0.3 μmole of xylulose, 0.4 μmole of ATP, and 0.01 mg. of xylulose kinase in 0.68 ml. of 0.1 M Tris buffer, pH 7.5. Sulfhydryl compounds did not increase the rate.

Preparation and Properties of D-Xylulose 5-Phosphate

When the purified kinase preparations were incubated with D-xylulose and ATP, a mixture of xylulose phosphate and ribulose phosphate resulted, indicating the presence of PKPE. The mixture of xylulose phosphate and ribulose phosphate was separated from other reaction products by ion exchange chromatography, but these two components could not be separated from each other. In order to obtain the xylulose ester free of ribulose phosphate, the mixture of the two esters was further incubated with an excess of ATP and phosphoribulokinase (13), which converts Ru-5-P to the diphosphate ester. From this final mixture Xu-5-P is readily isolated by ion exchange chromatography.

The initial reaction mixture contained 8 mg. of xylulose kinase (105 units), 450 μmoles of D-xylulose, 480 μmoles of ATP, 100 μmoles of cysteine, in 15 ml. of 0.05 M $NaHCO_3$, containing 0.01 M $MgCl_2$. The gas phase was 95 per cent nitrogen and 5 per cent carbon dioxide; the temperature, 37°. The reaction was followed manometrically in the Warburg apparatus until the evolution of carbon dioxide ceased, indicating the completion of the phosphorylation reaction. The reaction mixture was cooled to 4° and placed on a 12 cm. × 2.5 sq. cm. Dowex 1-formate column, washed with

25 ml. of water, and eluted by gradient elution with 200 ml. of water in the mixing chamber and 0.4 N formic acid containing 0.1 M sodium formate in the reservoir. The volume of each fraction collected was 10 ml. The fractions containing the phosphate esters (Fractions 32 to 42) were adjusted to pH 6.2 with 8 ml. of 0.6 N barium hydroxide and 25 ml. of 2 N KOH and precipitated with 4 volumes of ethanol. The precipitate was kept at 0° for 30 minutes, collected by centrifugation, washed with 10 ml. of 70 per cent ethanol, and dried *in vacuo*. The yield based on organic phosphate was 390 μmoles of ester phosphate, containing approximately 175 μmoles of D-xylulose 5-phosphate.

The barium salts were dissolved in 5 ml. of 0.05 N acetic acid, 0.8 ml. of 0.57 M Na_2SO_4 was added, and the barium sulfate removed by centrifugation. The phosphate esters were then incubated with 20 ml. of 0.05 M $NaHCO_3$ containing 0.01 M $MgCl_2$, 100 μmoles of cysteine, 250 μmoles of ATP, and 0.03 ml. of phosphoribulokinase (120 units, 0.48 mg. of protein). The chromatographic procedure used in separating the mixed esters was the same as that employed above. The fractions containing xylulose 5-phosphate (Fractions 30 to 34) were pooled and adjusted to pH 6.2 with 6 ml. of 0.6 N barium hydroxide and 13 ml. of 2 N KOH and precipitated with 4 volumes of ethanol. The precipitate was collected by centrifugation, washed with ethanol, and dried *in vacuo*. The yield based on organic phosphate was 167 μmoles.

The ketopentose moiety was characterized as follows: The barium salt was converted to the sodium salt with sodium sulfate and 6 μmoles were hydrolyzed with potato phosphatase as described in "Methods." The dephosphorylated product was assayed directly by employing (*a*) xylulose kinase and the ADP spectrophotometric assay system, and (*b*) the cysteine-carbazole reaction as modified by Ashwell and Hickman (6) for estimating the per cent of xylulose in the dephosphorylated reaction mixture. According to the former method xylulose accounted for 82 per cent of the organic phosphate, while the colorimetric assay showed 96 per cent of the ketopentose to be xylulose. (*c*) Paper chromatography as described in "Methods" revealed only one component in the dephosphorylated product, having the same R_F as authentic xylulose and giving the characteristic gray-blue color when sprayed with the TCA-orcinol reagent. (*d*) A comparison of the absorption spectra given by authentic xylulose and the dephosphorylated product in the orcinol reaction is shown in Fig. 1. The curves obtained with xylulose and the product of dephosphorylation were very similar, but quite different from that given by ribulose. For xylulose 5-phosphate the absorption at 540 mμ was considerably depressed. The extinction coefficient at 670 mμ was somewhat higher than that of free xylulose. (*e*) As indicated previously, the rate of color development in

the cysteine-carbazole reaction is characteristic for xylulose, which requires more than an hour for full development and differentiates it from ribulose, which is completely converted in less than 10 minutes. The dephosphorylated product behaves quantitatively like xylulose in this test (Fig. 2).

Periodate oxidation of the phosphate ester gave somewhat low results, only 67 per cent of the theoretical quantity of periodate being consumed.
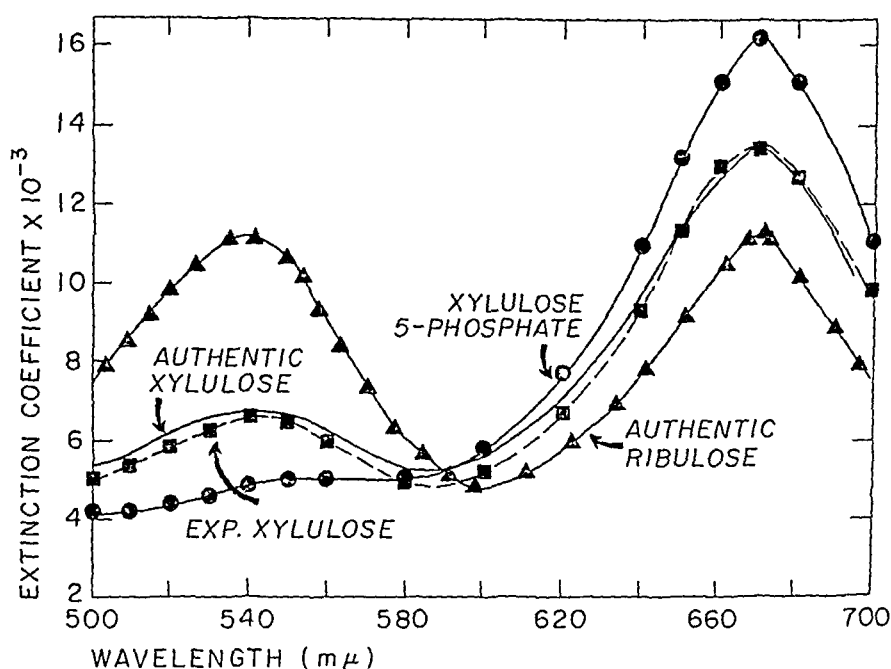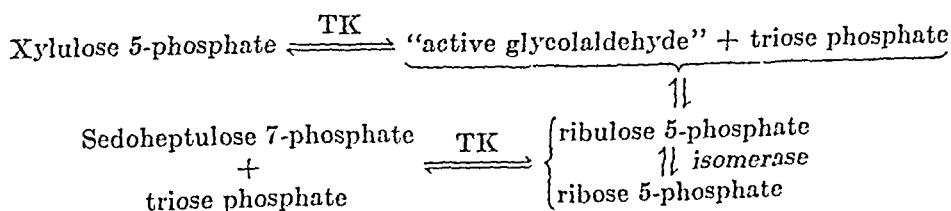

FIG. 1. Absorption spectra in the orcinol reaction. Xylulose 5-phosphate was the chromatographed reaction product. Experimental xylulose was the same substance after enzymatic dephosphorylation.

Activity of Xylulose 5-Phosphate with Transketolase—Since transketolase catalyzes the transfer of "active glycolaldehyde" from a number of ketol compounds to suitable acceptors, it was of interest to compare the reactivity of D-xylulose 5-phosphate and D-ribulose 5-phosphate, which differ only in the position of the hydroxyl group on the 3rd carbon atom.

Several assay systems were employed to test the reactivity of these substrates. The first may be described by the accompanying equations.

$$\text{Xylulose 5-phosphate} \underset{\text{TK}}{\rightleftarrows} \text{"active glycolaldehyde"} + \underbrace{\text{triose phosphate}}$$

$$\underbrace{\begin{array}{l}\text{Sedoheptulose 7-phosphate} \\ + \\ \text{triose phosphate}\end{array}}_{} \underset{\text{TK}}{\rightleftarrows} \left\{\begin{array}{l}\text{ribulose 5-phosphate} \\ \quad\updownarrow \ isomerase \\ \text{ribose 5-phosphate}\end{array}\right.$$

With the formation of triose phosphate as a measure of substrate activity, it was found that xylulose 5-phosphate was a good substrate for trans-
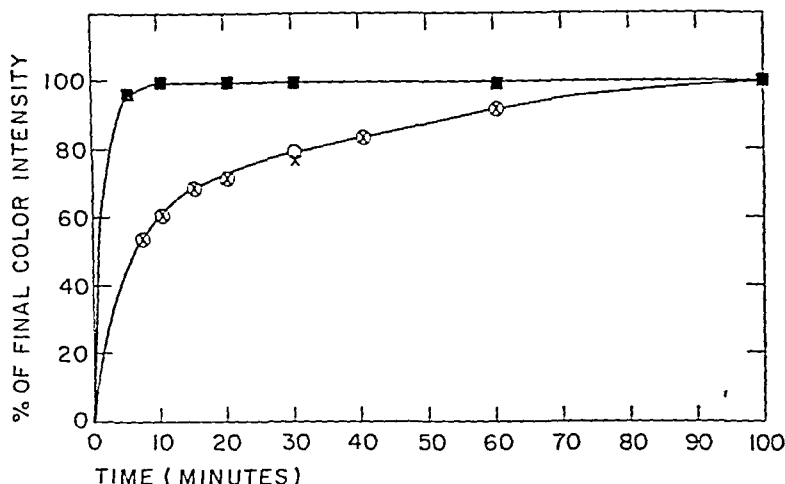

Fig. 2 Rate of color development in the cysteine-carbazole reaction. O represents an authentic sample of xylulose; X, the dephosphorylated product of ketopentose phosphate prepared by the enzymatic phosphorylation of xylulose; ■, authentic sample of ribulose.
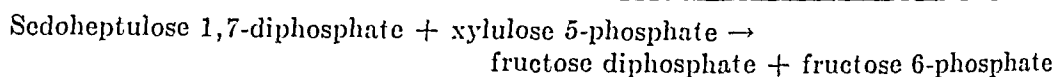
TABLE II

Pentose Phosphates As Substrates for Transketolase

Substrate	Triose phosphate formed per 10 min
	$\mu mole \times 10^{-2}$
Ribose 5-phosphate	3.1
Ribulose 5-phosphate	3.4
Xylulose 5-phosphate	1.9

The assay system contained 0 5 μmole of pentose phosphate, 0 01 ml. of DPNH, 1 mg of glycerophosphate dehydrogenase, 0 9 ml. of 0 01 M glycylglycine-cysteine buffer at pH 7 5, 3 units of spinach transketolase (contains phosphoriboisomerase), and 1 μmole of MgCl₂ The oxidation of DPNH was measured at 340 mμ in a 1 ml. cuvette. The temperature was 25°.

ketolase (Table II).   To confirm these findings, the reaction mixture was dephosphorylated, chromatographed on paper, and sprayed with the TCA-orcinol reagent.   In the complete system the formation of triose phosphate was accompanied by the appearance of a large spot having the characteristic light blue color of heptuloses and the same R_F as sedoheptulose.   No heptulose spot could be detected in the control with boiled transketolase.

In the second system employed, transketolase activity was measured in the following manner.

$$\text{Sedoheptulose 1,7-diphosphate} \xrightleftharpoons{\text{aldolase}}$$

$$\text{tetrose 4-phosphate triose} + \text{phosphate}$$

$$\text{Xylulose 5-phosphate} \xrightleftharpoons{\text{TK}} \text{``active glycolaldehyde''} + \text{triose phosphate}$$

$$\text{``Active glycolaldehyde''} + \text{tetrose phosphate} \xrightleftharpoons{\text{TK}} \text{fructose 6-phosphate}$$

$$2 \text{ triose phosphate} \xrightleftharpoons{\text{aldolase}} \text{fructose diphosphate}$$

Sedoheptulose 1,7-diphosphate + xylulose 5-phosphate →
fructose diphosphate + fructose 6-phosphate
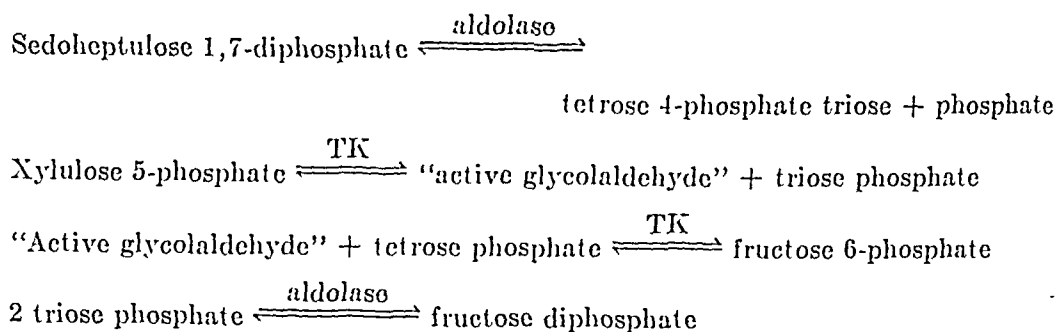
Hexose monophosphate formation was followed by the reduction of TPN to TPNH in the presence of glucose 6-phosphate dehydrogenase and glucose phosphate isomerase (Fig. 3). The rate of reaction with xylulose phosphate was somewhat greater than with ribulose phosphate, although the reaction stopped at about 63 per cent of the expected completion.

Evidence for Phosphoketopentoepimerase

Crude extracts of *L. pentosus* were found to contain high transketolase activity, and it was postulated that the transformation of xylulose 5-phosphate to ribulose 5-phosphate was due to the presence of this enzyme. It was therefore assumed that inactivation of transketolase would permit xylulose 5-phosphate to accumulate. Accordingly, an experiment was carried out with a crude extract which had been heated at 55° for 5 minutes at pH 7.7. This treatment resulted in a 77 per cent loss in kinase activity and a complete loss in transketolase activity. In this experiment D-xylulose and ATP gave rise to a mixture of pentose phosphates, which was dephosphorylated with potato phosphatase and analyzed by the cysteine-carbazole reaction and by paper chromatography. The results always indicated a 45:55 mixture of xylulose 5-phosphate and ribulose 5-phosphate.

A more detailed study showed that a new enzyme, phosphoketopentoepimerase, was present which catalyzed the epimerization reaction. The preparation and properties of PKPE will now be considered.

Preparation—5 ml. of crude bacterial extract were treated with 0.05 volume of 1 M $MnCl_2$ as described previously. The suspension was centrifuged, and the clear supernatant solution was heated at 65° for 5 minutes and then fractionated with ammonium sulfate. 2 ml. of saturated ammonium sulfate were added, and the precipitate was discarded; 4.5 ml. of saturated ammonium sulfate were then added to the supernatant solution,
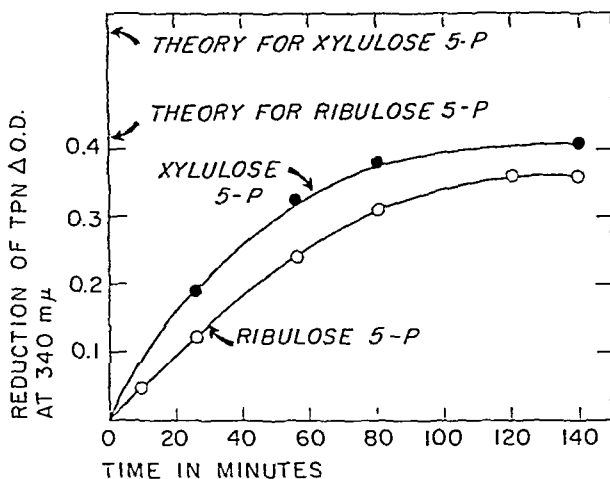
FIG. 3. The utilization of xylulose phosphate and ribulose phosphate by transketolase. The complete system contains 1 μmole of sedoheptulose diphosphate, 32 μmoles of triethylamine at pH 7.5, 8 μmoles of EDTA, 0.16 μmole of TPN, 0.12 mg. of aldolase, 1 unit of spinach transketolase, 1.1 mg. of muscle phosphohexoisomerase preparation, 0.2 mg. of *Zwischenferment*, 0.5 μmole of magnesium chloride, and 0.091 μmole of xylulose phosphate or 0.067 μmole of ribulose phosphate. See the text for a description of the reaction sequence.

TABLE III

Equilibration of Xylulose 5-Phosphate and Ribulose 5-Phosphate Catalyzed by Phosphoketopentoepimerase

Conditions	Substrate	Xylulose formed
		per cent
No enzyme	Ribulose 5-phosphate	0
Boiled enzyme	"	0
Enzyme	"	49
No enzyme	Xylulose 5-phosphate	94
Boiled enzyme	"	97
Enzyme	"	43

0.05 ml. of PKPE (0.9 mg. of protein per ml.), 0.11 μmole of xylulose 5-phosphate or 0.1 μmole of ribulose 5-phosphate, 0.1 ml. of 0.1 M Tris buffer at pH 7.5. Incubation time 20 minutes at 37°. At the end of the incubation, 0.02 ml. of 0.5 M acetate buffer at pH 5.2 was added. The mixture was heated to 100° for 5 minutes, cooled, and 0.01 ml. of potato phosphatase (10 units) was added. The system was incubated for 60 minutes at 37° and then tested directly with the cysteine-carbazole reagents. See "Methods" for additional details.

and the precipitate containing PKPE was collected and dissolved in 1 ml. of water. Since in some cases ATPase activity was still detectable, the final fraction was heated to 70° for 5 minutes. Although no attempt is made here to define the activity of PKPE in quantitative terms, the experiments to be described permit a partial characterization of the enzyme.
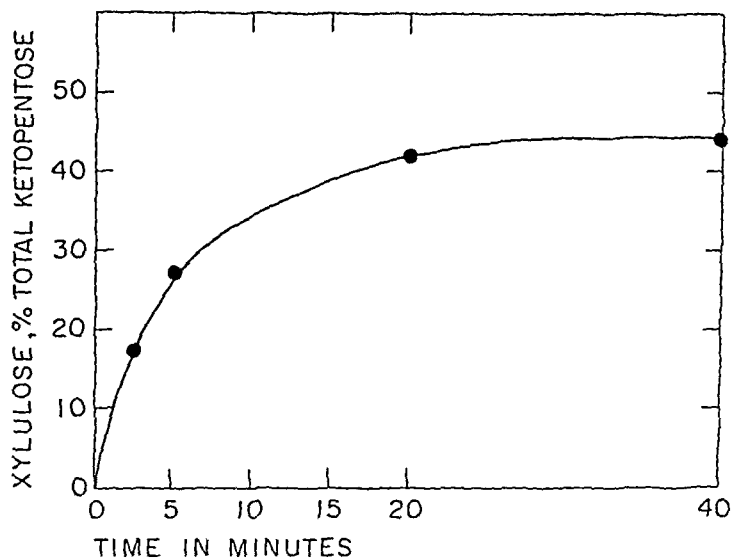

FIG. 4. Rate of conversion of ribulose phosphate to xylulose phosphate by PKPE. 0.05 ml. of PKPE (0.9 mg. per ml.), 0.25 μmole of ribulose phosphate, 10 μmoles of Tris buffer at pH 7.5, and H_2O to a final volume of 0.4 ml. The temperature was 37°. For further details of analysis see the legend to Table III.

Properties—With either ribulose 5-phosphate or xylulose 5-phosphate as substrate, an equilibrium mixture of both esters is rapidly attained (Table III).

$$\text{Xylulose 5-phosphate} \rightleftharpoons \text{ribulose 5-phosphate}$$
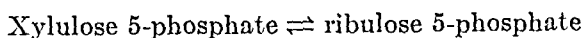
The equilibrium value for the ratio of ribulose 5-phosphate to xylulose 5-phosphate was approximately 1.20. Under the experimental conditions, with ribulose 5-phosphate as substrate the reaction had reached equilibrium in 20 minutes and was more than 60 per cent complete in 5 minutes (Fig. 4). The products of the reaction have been dephosphorylated and subjected to paper chromatography. In the experiment with ribulose 5-phosphate and boiled PKPE, no xylulose spot was present; with active PKPE, a strong xylulose spot was observed. No heptulose spot was detected The enzyme does not appear to require metal ions for activity. It was not inhibited by EDTA (final concentration 10^{-2} M), nor was it affected by the addition of sulfhydryl compounds such as cysteine or glutathione. Ribulose 1,5-diphosphate did not serve as a substrate.
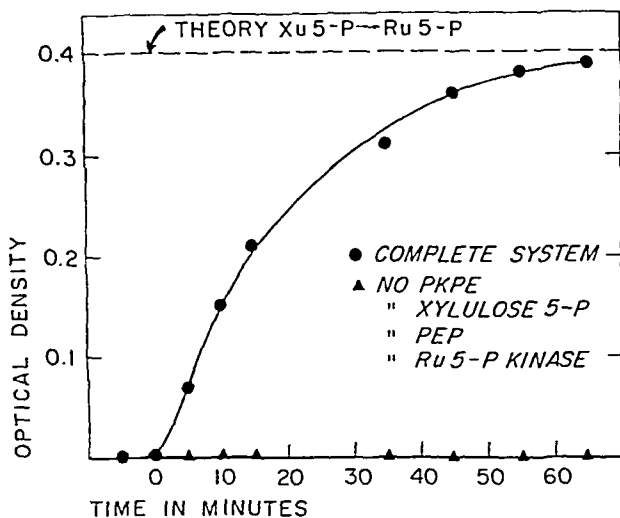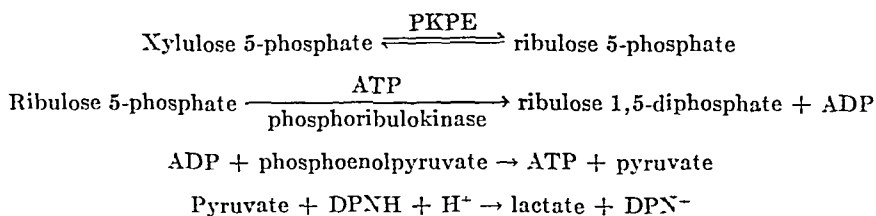
FIG. 5. Rate of DPNH oxidation as a function of the epimerization of xylulose phosphate to ribulose phosphate by PKPE. The complete system (1.5 ml., 25°) contained 0.45 μmole of ATP (Sigma, crystalline), 2 μmoles of cysteine at pH 7.5, 2 μmoles of magnesium chloride, 6 μmoles of phosphate buffer at pH 7.5, 0.6 μmole of phosphoenolpyruvate (PEP), 0.5 unit of pyruvate kinase, 2 units of lactic dehydrogenase, 0.24 μmole of DPNH, 0.065 μmole of xylulose 5-phosphate, 20 units of phosphoribulokinase, and 0.05 ml. of PKPE (0.9 mg. per ml.). Control reaction mixtures are indicated in the figure.

The epimerization can be studied spectrophotometrically with the aid of phosphoribulokinase and the ADP assay system.

$$\text{Xylulose 5-phosphate} \underset{}{\overset{\text{PKPE}}{\rightleftarrows} } \text{ribulose 5-phosphate}$$

$$\text{Ribulose 5-phosphate} \xrightarrow[\text{phosphoribulokinase}]{\text{ATP}} \text{ribulose 1,5-diphosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \rightarrow \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{DPNH} + \text{H}^+ \rightarrow \text{lactate} + \text{DPN}^-$$

As indicated in Fig. 5, no oxidation of DPNH occurred in the absence of PKPE, phosphoenolpyruvate, or phosphoribulokinase. In the presence of the ribulose 5-phosphate trapping system, all of the xylulose 5-phosphate is utilized. No transketolase activity could be detected in the PKPE system by the usual assay procedures.

DISCUSSION

With the identification of xylulose kinase and PKPE in extracts of *L. pentosus*, it is now possible to formulate a sequence of reactions leading

from xylose to ribose 5-phosphate. A (xylose isomerase) has been found in *L. pentosus* (2), in *P. hydrophila* (3), and recently in *P. pestis* (4). B

ribose 5-phosphate

$$E \parallel \overset{D}{\rightleftharpoons} \text{sedoheptulose 7-phosphate} + \text{triose phosphate}$$

ribulose 5-phosphate

$$\text{Xylose} \underset{A}{\rightleftharpoons} \text{xylulose} \xrightarrow[B]{\text{ATP}} \text{xylulose 5-phosphate} \quad \overset{C}{\diagup} \quad D \parallel$$
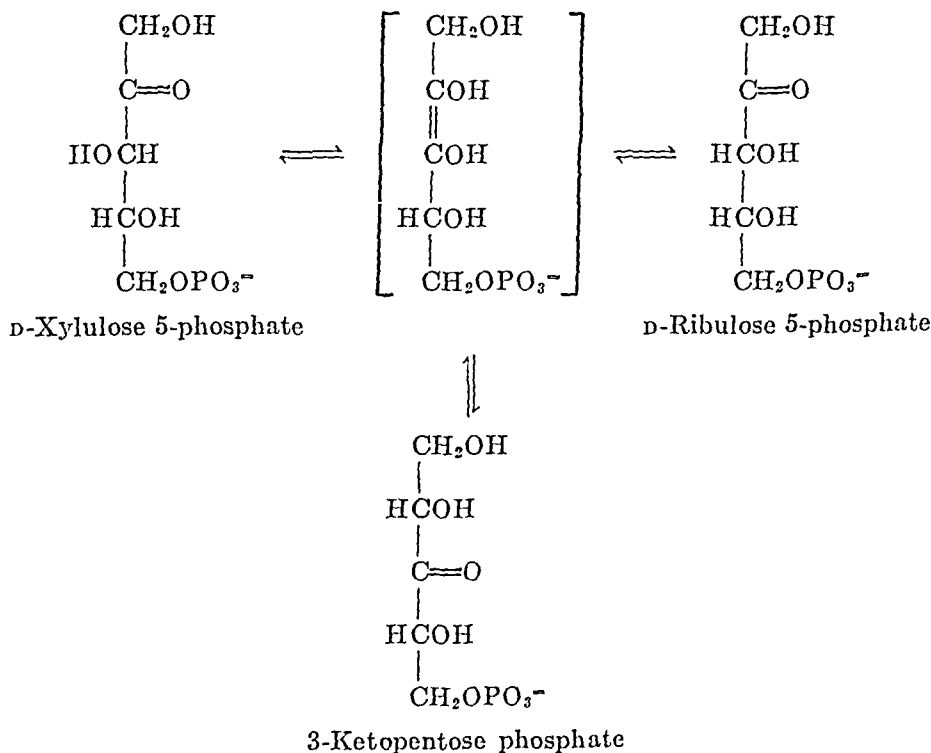
$$D \diagdown$$

$$(\text{C}_2 + \text{triose phosphate})$$
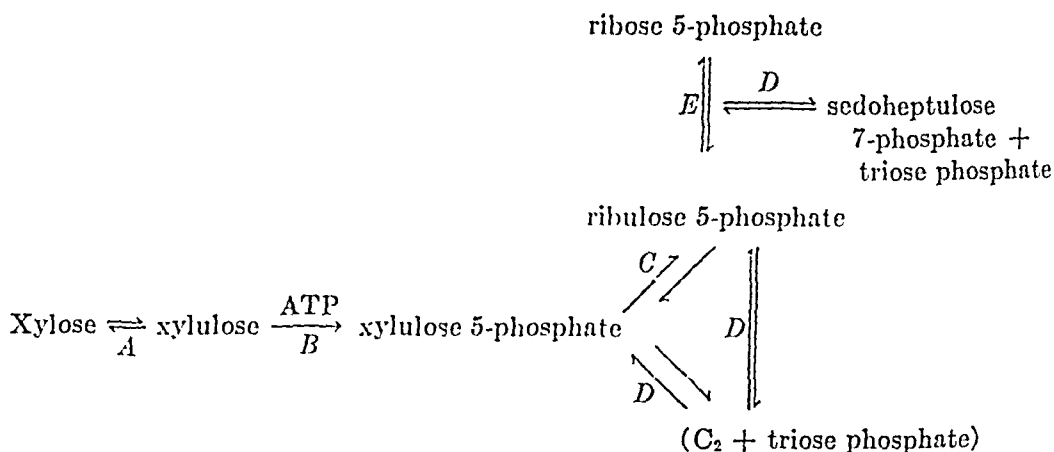
(xylulose kinase) has been described in some detail in this paper. The enzyme is highly specific and catalyzes the phosphorylation of D-xylose by ATP to yield xylulose 5-phosphate. This ester participates in at least two reactions. It can be converted to ribulose 5-phosphate by the relatively stable enzyme PKPE (C). This enzyme not only occurs in extracts of *L. pentosus*, but has also been found in spleen extracts (6) and in

$$
\begin{array}{ccc}
\text{CH}_2\text{OH} & \left[\ \text{CH}_2\text{OH}\ \right] & \text{CH}_2\text{OH} \\
| & | & | \\
\text{C}{=}\text{O} & \text{COH} & \text{C}{=}\text{O} \\
| & \| & | \\
\text{HOCH} \rightleftharpoons & \text{COH} \rightleftharpoons & \text{HCOH} \\
| & | & | \\
\text{HCOH} & \text{HCOH} & \text{HCOH} \\
| & | & | \\
\text{CH}_2\text{OPO}_3{}^- & \left[\ \text{CH}_2\text{OPO}_3{}^-\ \right] & \text{CH}_2\text{OPO}_3{}^-
\end{array}
$$

D-Xylulose 5-phosphate   D-Ribulose 5-phosphate

$$
\begin{array}{c}
\text{CH}_2\text{OH} \\
| \\
\text{HCOH} \\
| \\
\text{C}{=}\text{O} \\
| \\
\text{HCOH} \\
| \\
\text{CH}_2\text{OPO}_3{}^-
\end{array}
$$

3-Ketopentose phosphate

muscle fractions.[4] The mechanism of epimerization is as yet unknown. However, on the basis of evidence for a 3-ketopentose phosphate in their reaction products, Ashwell and Hickman (6) have postulated the sequence represented in the accompanying diagram. With the *L. pentosus* PKPE preparations no evidence for the 3-keto sugar is available.

An additional possibility for epimerization is the observation that xylulose 5-phosphate serves as a substrate for transketolase (*D*). As yet the experimental conditions have not been found for the accumulation of ribulose 5-phosphate from a reaction involving xylulose 5-phosphate and transketolase. With the purified spinach preparations sufficient phosphoriboisomerase is present to produce ribose 5-phosphate, which serves as an acceptor in the transketolase reaction. Thus, the formation of sedoheptulose 7-phosphate is always observed, whether the initial substrate is xylulose 5-phosphate or ribulose 5-phosphate.

In xylose-adapted *L. pentosus* cells these reactions are involved in the utilization of xylose (1, 2). It is of interest that they are also present in mammalian cells. This circumstance suggests that the xylulose ester may play a wider rôle in carbohydrate metabolism than was hitherto suspected.

SUMMARY

Xylulose kinase has been isolated from xylose-adapted *Lactobacillus pentosus* cells and has been purified some 50-fold. Of the several substrates tested, only D-xylulose is reactive. The enzyme is metal-activated.

The kinase was employed for the preparation of D-xylulose 5-phosphate. Xylulose 5-phosphate participates as substrate with two enzymes: (*a*) transketolase, which together with phosphoriboisomerase converts xylulose 5-phosphate to heptulose 7-phosphate, and (*b*) a newly described enzyme, phosphoketopentoepimerase, which catalyzes the epimerization of xylulose 5-phosphate to ribulose 5-phosphate.

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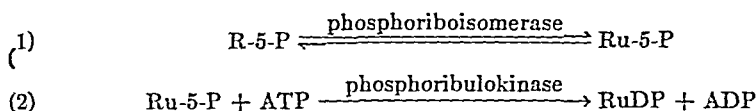
SPINACH PHOSPHORIBULOKINASE

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It was observed by Benson (1) that a compound with the properties of ribulose diphosphate is among the early products of photosynthesis. The importance of this compound in the CO_2 fixation process was indicated by the kinetic studies of Calvin and his coworkers (2, 3) and strongly supported by the finding that algal extracts would fix CO_2 in the absence of light when RuDP[1] was added (4). Independently, Weissbach et al. (5) observed that a soluble extract of spinach leaves was capable of forming carboxyl-labeled PGA from $C^{14}O_2$ in the presence of R-5-P. Fractionation of the spinach extracts has yielded an enzyme system which forms RuDP from R-5-P and ATP (6). In the present communication it will be demonstrated that the formation of RuDP is due to two enzymes which catalyze the following reactions:

(1) $$\text{R-5-P} \xrightleftharpoons{\text{phosphoriboisomerase}} \text{Ru-5-P}$$

(2) $$\text{Ru-5-P} + \text{ATP} \xrightarrow{\text{phosphoribulokinase}} \text{RuDP} + \text{ADP}$$

The first of these enzymes, phosphoriboisomerase, has been reported in yeast preparations (7) and purified from alfalfa (8). Both this enzyme and phosphoribulokinase have now been purified from spinach leaf extracts.

Methods

The following substances were obtained from commercial sources: crystalline ATP (Sigma Chemical Company and Pabst Laboratories),

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† Fellow of The National Foundation for Infantile Paralysis.

[1] The following abbreviations are used: RuDP, ribulose 1,5-diphosphate; R-5-P, ribose 5-phosphate; Ru-5-P, ribulose 5-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN, diphosphopyridine nucleotide; DPNH, dihydrodiphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; PGA, 3-phosphoglyceric acid; S-7-P, sedoheptulose 7-phosphate; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; UTP, uridine triphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; GSH, glutathione; TEA, triethanolamine; P, phosphate.

ADP, TPN (80 per cent pure), and hexokinase (Sigma Chemical Company), and R-5-P (Schwarz Laboratories). S-7-P was prepared as previously described (9); DPN was prepared by the method of Kornberg and Pricer (10), and DPNH by the method of Ohlmeyer (11). Phosphoenolpyruvate was a generous gift of W. E. Pricer, Jr., of this Institute. 5-Phosphoribonic acid was prepared from R-5-P by bromine oxidation (12).

Two preparations of Ru-5-P were used during the course of this work. One preparation was obtained by the enzymatic oxidation of 6-phosphogluconate as previously described (13). The product had a specific rotation of $[\alpha]_D^{20}$ $-29.6°$ (c 2.83 in 0.2 N HCl)[2] and was contaminated with small amounts of R-5-P and 6-phosphogluconate. On a weight basis the product was 70 per cent pure.

The second preparation was derived from R-5-P by treatment with purified spinach phosphoriboisomerase. At 60°, the equilibrium mixture contained about 55 per cent of the total pentose phosphate as Ru-5-P, as measured with the modification of the cysteine-carbazole method (14). After incubation at this temperature, residual R-5-P was oxidized with bromine at pH 5.0, and the products were separated by chromatography on a Dowex 1 Cl⁻ column. Ru-5-P was eluted with 0.02 N HCl. Large losses were sustained during the bromine oxidation, and the over-all yield based on R-5-P was only 16 per cent. On a weight basis, the purity was 25 per cent. On paper chromatography following hydrolysis of the product with potato phosphatase[3] the only sugar detected was ribulose.

Pentose was measured by the method of Mejbaum (15) with a 40 minute heating period. Phosphate was determined by the method of Fiske and Subbarow (16). Lactic dehydrogenase was prepared according to Kornberg and Pricer (17). The final preparation contained 2500 units per ml. Phosphoenolpyruvic kinase was purified from rabbit muscle by the method of Kornberg and Pricer (17) through the calcium phosphate step. The preparation at this stage contained 81.3 units per ml., with a specific activity of 24.3 units per mg. ADP was determined as described by these authors (17); ATP was measured in the hexokinase-*Zwischenferment* assay (18).

Phosphoriboisomerase

Assay—The procedure employed was a modification of that of Axelrod and Jang (8). The reaction mixture, containing 2 μmoles of R-5-P, 30

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[2] The rotation measurements are based on pentose determinations by the orcinol method, compared to a standard arabinose solution and uncorrected for the relatively low values given by ketopentoses.

[3] Prepared by an unpublished procedure of Dr. A. Kornberg.

μmoles of Tris buffer, pH 7.1, 1 μmole of cysteine, and suitable dilutions of enzyme (diluted with 0.1 M Tris buffer, pH 7.1) in a total volume of 0.8 ml., was incubated at 38° for 10 minutes. Ru-5-P was determined in the cysteine-carbazole test. A unit of enzyme was defined as the amount which catalyzes the formation of 1 μmole of Ru-5-P under the conditions described above. Protein was determined by the phenol method as described by Sutherland *et al.* (19).

Purification—The isomerase was purified from spinach leaves, which Axelrod *et al.* (20) have shown to contain high concentrations of this enzyme. 475 gm. of spinach leaves were homogenized for 3 minutes in a large Waring blendor with 1900 ml. of H_2O. The entire homogenate (2000 ml.) was brought to 0.4 saturation with $(NH_4)_2SO_4$ (462 gm.) and centrifuged for 15 minutes in an International PR-2 centrifuge. The cloudy supernatant solution was filtered through Schleicher and Schuell No. 588 fluted filter paper (Table I, "crude extract"). The filtrate (1900 ml.) was brought to 0.7 saturation with $(NH_4)_2SO_4$ (346 gm.), centrifuged, and the precipitate dissolved in water to give a total volume of 310 ml. ("Ammonium Sulfate I"). The green solution was heated to 60° by immersion in a 90° bath. As soon as the solution reached 60° (about 1.5 minutes), it was transferred to a 60° bath, kept at this temperature for 10 minutes, and then rapidly cooled in an ice-water mixture. The coagulated protein was removed by centrifugation (Servall SS-1 for 25 minutes) and washed with 20 ml. of water. The supernatant solution and washing were combined ("heated solution") and dialyzed for 48 hours against cold, flowing distilled water, and the heavy precipitate which formed was discarded. The straw-colored supernatant solution (400 ml., "dialysate") was brought to 0.5 saturation with $(NH_4)_2SO_4$ (116 gm.), centrifuged, and the precipitate discarded. The supernatant solution (500 ml.) was brought to 0.9 saturation (133 gm. of $(NH_4)_2SO_4$) and the precipitate dissolved in water ("Ammonium Sulfate II"). A summary of the purification procedure appears in Table I. The apparent increase in total activity may be due to removal of enzymes catalyzing competing reactions, particularly those which utilize Ru-5-P.

The final preparation was free of transketolase and phosphoribulokinase. It was moderately stable in the frozen state, losing approximately 50 per cent of its activity in 1 month at −10°. The equilibrium mixture formed at 38° in the presence of the enzyme contained 70 per cent of R-5-P and 30 per cent of Ru-5-P,[4] in excellent agreement with the result obtained with the yeast and alfalfa isomerases (7, 8).

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[4] R-5-P disappearance was determined directly as well as Ru-5-P formation. The authors are indebted to Dr. G. Ashwell for the R-5-P analyses.

Phosphoribulokinase

Assay—For routine enzyme assay, R-5-P was the substrate and an excess of phosphoriboisomerase was added. Under these conditions, the rate of reaction could be followed by the appearance of alkali-labile phosphate, since both phosphate groups of RuDP are alkali-labile, and R-5-P, ATP, and ADP do not yield inorganic phosphate. The assay mixture contained 8 μmoles of R-5-P, 10 μmoles of ATP, 10 μmoles of MgCl$_2$, 2 μmoles of cysteine, 40 μmoles of TEA buffer, pH 8.3, 50 units of phosphoriboisomerase, and the enzyme preparation to be assayed in a total volume of 1 ml. The reaction was carried out at 38° for 10 minutes and stopped by heating in a boiling water bath for 1.5 minutes. An aliquot was transferred to 1 N NaOH, incubated at room temperature for 20 min-

TABLE I

Purification of Phosphoriboisomerase

Fraction	Total volume	Total units,* $\times 10^{-6}$	Specific activity
	ml.		*units per mg.*
Crude extract..................	1900	1.16	47
Ammonium Sulfate I..........	310	0.95	124
Heated......................	329	2.55	1730
Dialysate....................	400	2.4	2400
Ammonium Sulfate II..........	41.5	1.6	3000

* Micromoles of Ru-5-P formed in 10 minutes.

utes, and inorganic P determined. In order to correct for the formation of Ru-5-P, which yields approximately 50 per cent of its phosphate as inorganic phosphate under these conditions, a control was run with phosphoriboisomerase alone, and the alkali-labile phosphate formed was subtracted from the test values.

An alternative assay involved the determination of ADP with phosphoenolpyruvic kinase and lactic dehydrogenase (17). Pyruvic acid formed from phosphoenolpyruvate in this system was determined spectrophotometrically with DPNH. No reaction occurred in the absence of ADP. Controls were run in order to correct for ATPase activity, which was relatively low in these spinach extracts. The reaction velocity was proportional to enzyme concentration with either assay (Fig. 1).

In the course of purification, two procedures were employed for protein determination. The Bücher procedure (21) was used for rapid protein determinations. However, to insure accurate estimation of protein concentration with highly colored protein solutions, the method of Sutherland *et al.* was used (19).

Purification—Spinach leaves from a local market were washed with cold tap water and the stems discarded. An acetone powder was prepared by three extractions with acetone (previously cooled to −20°). The first extraction was carried out with 3 volumes of acetone in a Waring blendor for 3 minutes. For the subsequent extractions 1 volume of acetone was used. Each suspension was filtered rapidly on a large Büchner
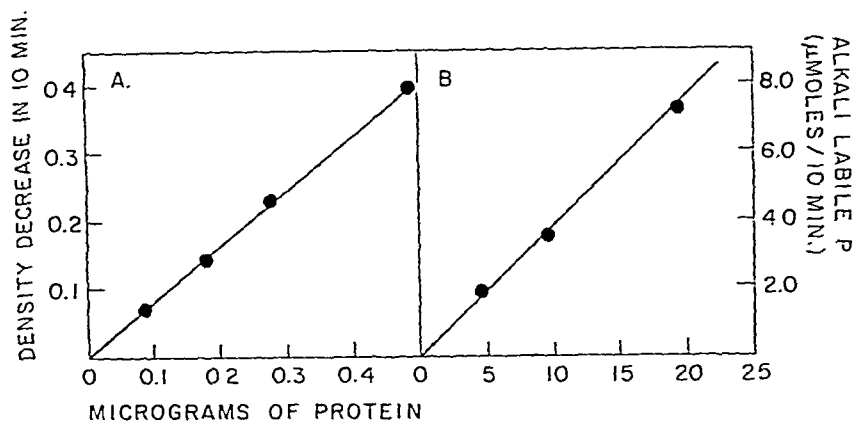

Fig. 1. Effect of enzyme concentration on reaction rate. For the spectrophotometric assay (*A*) the reaction mixture contained 0.5 μmole of Ru-5-P, 3 μmoles of ATP, 50 μmoles of potassium phosphate buffer, pH 7.9, 2 μmoles of cysteine, 5 μmoles of Mg^{++}, 0.6 μmole of phosphoenolpyruvate, 2.1 units of lactic dehydrogenase, 0.5 unit of phosphopyruvic kinase, 0.06 μmole of DPNH, and varying amounts of phosphoribulokinase. The total volume was 1.5 ml. and the light path 1.0 cm. For the alkali-labile P assay (*B*) the reaction mixture contained 8 μmoles of R-5-P, 10 μmoles of ATP, 10 μmoles of Mg^{++}, 6 μmoles of cysteine, 40 μmoles of TEA, pH 8.3, 50 units of isomerase, and kinase in a total volume of 1.0 ml. The mixture was incubated 10 minutes at 38°; the reaction was stopped by heating at 100° for 1.5 minutes. An aliquot was removed, made 1 N with NaOH, neutralized after 20 minutes, and inorganic P determined.

funnel. The light green powder which remained was stored in a desiccator under vacuum.

50 gm. of acetone powder were extracted for 10 minutes with gentle stirring with 1800 ml. of 2 × 10^{-3} M TEA buffer, pH 8.5. The mixture was centrifuged and the supernatant solution decanted through glass wool (Table II, "Crude extract," 1340 ml.). The extract was treated with 260 gm. of ammonium sulfate and the precipitate discarded. Ammonium sulfate (310 gm.) was added to the supernatant solution (1420 ml.) and the precipitate collected and dissolved in 150 ml. of 2 × 10^{-3} M TEA buffer, pH 8.3. The protein content was determined by the Bücher turbidimetric procedure and the solution diluted with water to a protein

content of 5 mg. per ml. ("Ammonium Sulfate I," 1224 ml.). This solution was usually about 5 per cent saturated with respect to ammonium sulfate (determined with a Barnstead purity meter). Solid ammonium sulfate (217 gm.) was added to give 0.40 saturation. Following centrifugation, the supernatant solution (1270 ml.) was brought to 0.55 per cent saturation by the addition of 113 gm. of $(NH_4)_2SO_4$. The precipitate was dissolved in 100 ml. of 2×10^{-3} M triethanolamine buffer, pH 8.5 ("Ammonium Sulfate II," 113 ml.). Water was added to bring the protein content to 14 mg. per ml., and an equal volume of 0.1 M sodium acetate, pH 7.0, was added. 210 ml. of acetone, cooled to $-20°$, were added slowly while the solution was cooled in a $-8°$ bath. The heavy precipitate which formed was removed by centrifugation at $-10°$. The supernatant solution (468 ml.) was treated with acetone (212 ml.) and centri-

TABLE II

Purification of Phosphoribulokinase

Fraction	Total units*	Specific activity
		units per mg.
Crude extract........	31,000	6.2
Ammonium Sulfate I...............	42,000	20
" " II...............	43,000	62
Acetone...........................	40,000	300
Ammonium Sulfate III.............	40,000	310

* A unit is defined as that amount of enzyme which forms 1 μmole of alkali-labile P in 10 minutes at 38° under the test conditions specified in the text.

fuged as before. The precipitate was extracted with 125 ml. of 0.1 M Tris buffer, pH 7.7, and the residue discarded ("Acetone," 136 ml.). To concentrate the enzyme, the acetone fraction was treated with 59.5 gm. of ammonium sulfate and the precipitate collected and dissolved in 10 ml. of 0.1 M Tris buffer, pH 7.7 ("Ammonium Sulfate III," 14 ml.).

The final phosphoribulokinase preparation was stable in the frozen state at $-10°$, but lost activity on repeated freezing and thawing. It was found to contain 300 units of phosphoriboisomerase and 0.4 unit of transketolase (9) per ml. Neither CO_2 fixation nor RuDP phosphatase activity[5] could be detected.

Properties of Phosphoribulokinase

Effect of pH—The phosphorylation reaction proceeds at maximal velocity at pH 7.9 (Fig. 2). Above or below this pH the rate of the reaction declined sharply.

[5] We are indebted to Dr. Howard H. Hiatt for this determination.

Pentose Phosphate Specificity—The purified kinase preparations utilized R-5-P very slowly unless phosphoriboisomerase was added (Fig. 3). This was in contrast to the behavior toward Ru-5-P, which was rapidly esterified in the absence of the isomerase. Under the conditions of these experi-
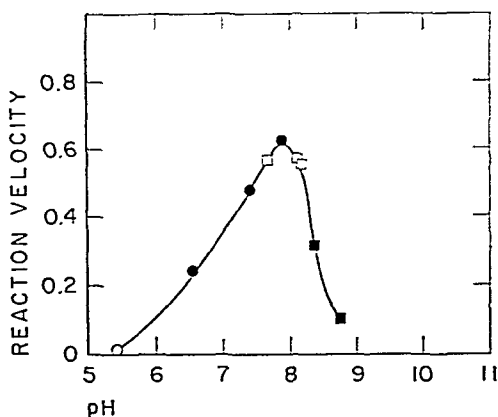

FIG. 2. pH optimum of phosphoribulokinase. The reaction mixture contained 40 μmoles of buffer, 1.5 μmoles of Ru-5-P, 3 μmoles of ATP, 5 μmoles of MgCl$_2$, 1 μmole of EDTA, and 0.43 unit of enzyme, specific activity 170, in a total volume of 1.0 ml. After 10 minutes at 38° the reaction was stopped by heating in a boiling water bath for 3 minutes. Aliquots (0.1 ml.) were assayed for ADP, as described in the legend to Fig. 1, except that Ru-5-P and ATP were omitted. The symbols represent acetate (O), phosphate (●), Tris (□), and TEA (■) buffers.
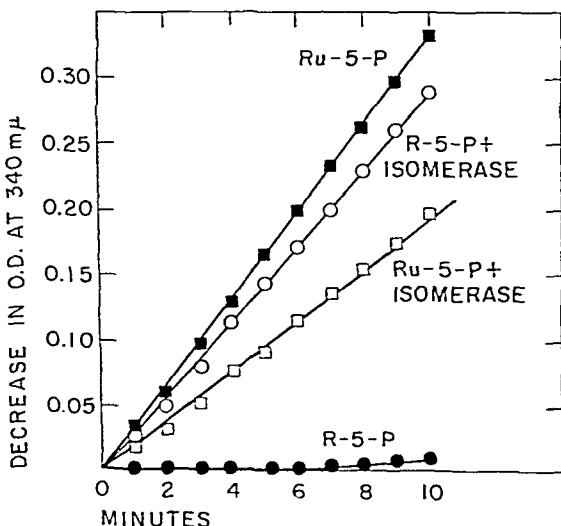

FIG. 3. Substrate specificity of phosphoribulokinase. The additions were as described in Fig. 1, except that 0.36 γ of protein, specific activity 440, was used.

ments, with Ru-5-P as the substrate, preincubation with isomerase resulted in a decrease in the reaction rate, since the concentration of substrate was reduced to suboptimal levels. At higher levels of protein the presence of traces of isomerase in the kinase preparation became evident and ultimately, when the protein concentration was increased 20-fold over that employed in Fig. 3, the rate of reaction with R-5-P became equal to that with Ru-5-P.

Further evidence against an initial phosphorylation of R-5-P was obtained with ribose 1,5-diphosphate.[6] This substance was not converted
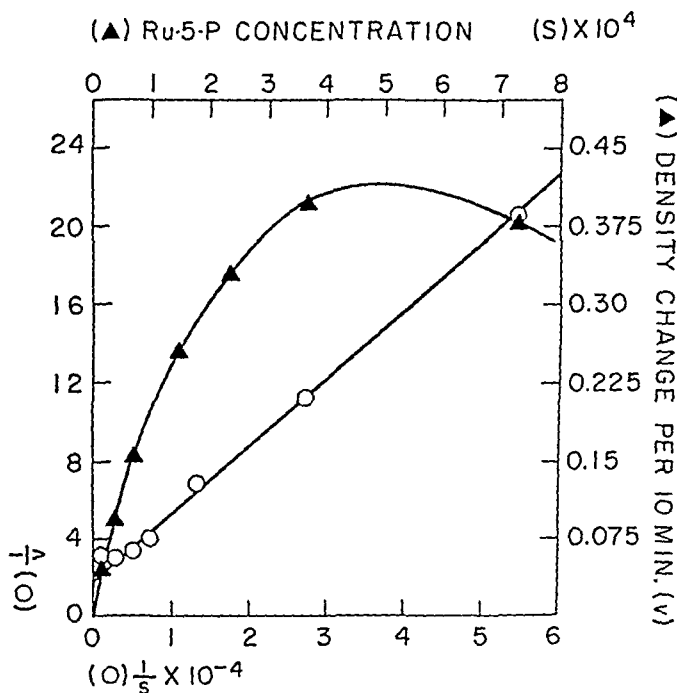

Fig. 4. Effect of Ru-5-P concentration. The spectrophotometric method described in the legend to Fig. 1, A was employed, except that 0.6 γ of kinase, specific activity 170, was used and 2 μmoles of EDTA were added instead of cysteine.

to RuDP by either the kinase or isomerase preparations and thus failed to meet the requirements for an intermediate in the formation of RuDP from R-5-P. As would be expected, the presence of a free aldehyde group in the 1 position appears to be necessary for isomerase activity.

No other substrates have been found to replace Ru-5-P as the phosphate acceptor. Neither fructose 6-phosphate nor sedoheptulose 7-phosphate was esterified. D-Xylulose 5-phosphate[7] was inactive with the kinase preparations. The absence of hexokinase was indicated by the failure to observe ADP formation with glucose as the substrate.

[6] Ribose 1,5-diphosphate was a generous gift of Mr. H. Klenow of the Universitetets Institut for Cytofysiologi, University of Copenhagen, Denmark.

[7] Kindly furnished by Dr. P. K. Stumpf.

Maximal activity was reached with Ru-5-P concentrations of about 3.7×10^{-4} M, and the Lineweaver-Burk plot (22) (Fig. 4) yielded a K_s value of 2.2×10^{-4} M. This relatively high affinity of the enzyme for its substrate permits the utilization of this reaction for the quantitative determination of Ru-5-P, although it is not completely specific, since, with the relatively large quantities of enzyme preparation required, R-5-P also reacts. The Ru-5-P preparations obtained by the action of isomerase on R-5-P were found to react as rapidly as those obtained by the oxidation of 6-phosphogluconate.

Nucleotide Specificity—Of a number of nucleotides tested, only ATP showed significant activity with phosphoribulokinase (Table III). However, this result is not unequivocal, since the reaction rates may be limited by the ability of the nucleoside diphosphate products to act as acceptors

TABLE III

Nucleotide Specificity for Phosphoribulokinase

Nucleotide	Quantity added	Activity, ΔD_{340} in 10 min.[*]
	μmoles	
ATP	2	0.470
ITP	2	0.019
UTP	1	0.013
CTP	1	0.004
GTP	1	0.006

[*] The assay conditions are the same as those in Fig. 1, *A*, with 0.5 μmole of Ru-5-P as substrate. UTP, CTP, GTP, and ITP were kindly supplied by Dr. L. A. Heppel of this Institute.

in the phosphoenolpyruvate assay system.[8] On the other hand, since the activity observed was only slightly greater than the blank value, it can tentatively be concluded that they are inactive in ribulose diphosphate formation. The inability of ADP to act as a phosphate donor was established by means of the manometric assay (23), which depends on acid formation and can be employed with either form of the nucleotide.

Maximal activity was reached when the ATP concentration was about 7×10^{-4} M and the K_s value, calculated from the Lineweaver-Burk plot (Fig. 5), was 2.8×10^{-4} M.

Metal Activation—The requirement for a divalent metal was evident even with the crude extract, and with purified preparations no activity could be demonstrated in the absence of the metal activator. Mg^{++}, which was the most effective ion tested, showed maximal activity at 5×10^{-3} M (Fig. 6). A number of ions including Mn^{++}, Ca^{++}, Co^{++}, and Cd^{++} replaced Mg^{++}, although these were considerably less effective.

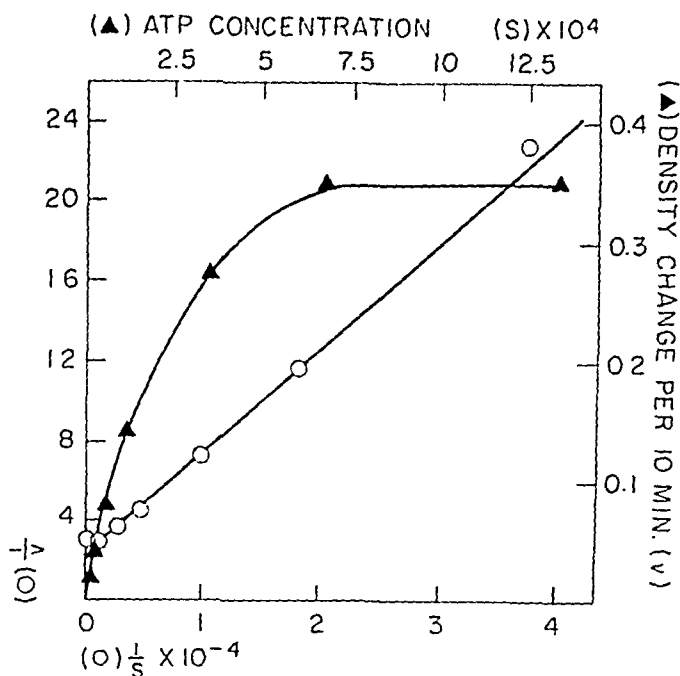[8] Unpublished observations of Dr. L. A. Heppel and Dr. J. Strominger.

FIG. 5. Effect of ATP concentration.   The assay conditions were as in Fig. 4, with 0.9 γ of phosphoribulokinase.
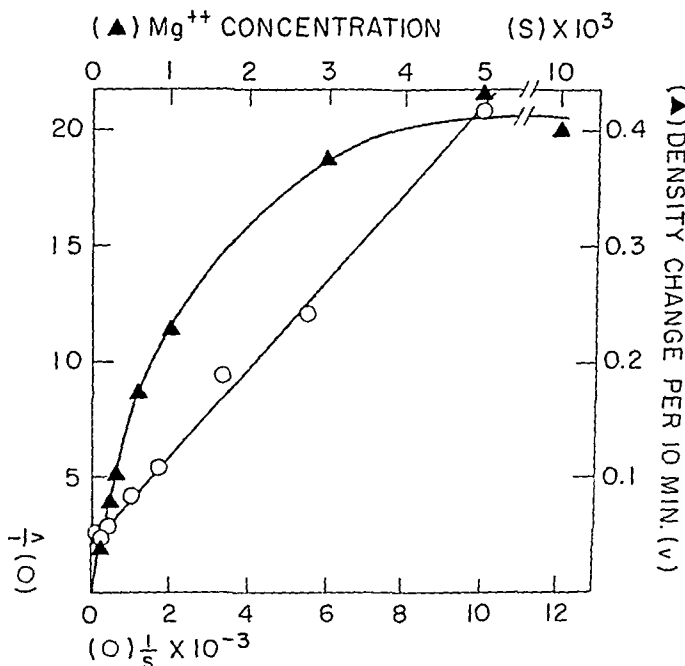

FIG. 6. Effect of Mg^{++} concentration.   The reaction mixture contained 1 μmole of Ru-5-P, 3 μmoles of ATP, 40 μmoles of Tris buffer, pH 7.9, 2 μmoles of GSII, 3 γ of phosphoribulokinase, specific activity 170, varying amounts of Mg^{++}, and H_2O to give a volume of 1 ml.   Aliquots were assayed for ADP as described in Fig. 1, A.

The requirement for Mg^{++} in the phosphopyruvic kinase assay for ADP made it necessary to carry out these determinations in two steps; the

TABLE IV

Metal Activation of Phosphoribulokinase

Metal	Concentration	ADP formed
	mole per l.	*μmole*
No metal		0
Mg	5×10^{-3}	0.252
Mn	5×10^{-3}	0.203
Ca	5×10^{-3}	0.162
Co	5×10^{-5}	0.048
Cd	5×10^{-3}	0.024
Zn	5×10^{-3}	0
Ni	5×10^{-3}	0
Ba	5×10^{-3}	0

The incubation mixture contained 1.5 μmoles of Ru-5-P, 3 μmoles of ATP, 40 μmoles of KPO_4 buffer, pH 7.9, 1 μmole of EDTA, 1.5 γ of protein, specific activity 170, metal salt added as chlorides or sulfates, and water to give a total volume of 1 ml. The reaction mixture was incubated at 38° for 10 minutes and the reaction stopped by heating for 3 minutes at 100°. An aliquot was removed and assayed for ADP formation (see Fig. 1, A).
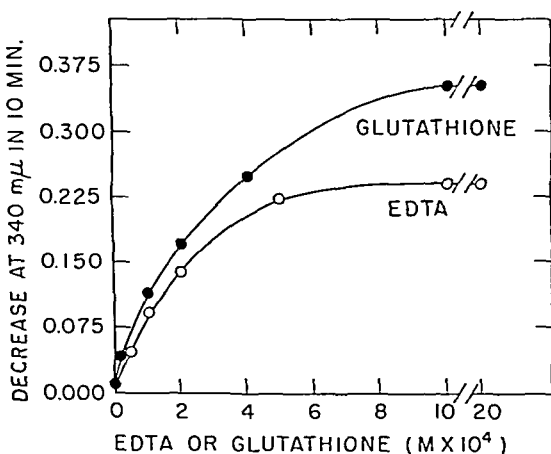

FIG. 7. Effect of GSH and EDTA. The assay conditions were as described in Fig. 1, A, with 0.9 γ of phosphoribulokinase, specific activity 170, in the case of GSH activation and 0.6 γ of kinase, specific activity 170, in the case of EDTA addition.

experimental procedure is indicated in Table IV. It was necessary to add EDTA in these experiments, since no activity was observed in its absence (see below).

Activation and Inhibition—Two lines of evidence suggested that phosphoribulokinase is a sulfhydryl enzyme. Fresh preparations were activated by the addition of EDTA or sulfhydryl compounds. In the ab-

TABLE V

Inhibition Studies with Phosphoribulokinase

Inhibitor	Concentration	Activator used	ΔD_{340} in 10 min.	Inhibition
	mole per l.			*per cent*
Cu^{++} *		GSH	0.294	
	5×10^{-6}	"	0.191	35
	1×10^{-5}	"	0.069	77
	1×10^{-4}	"	0.001	100
Hg^{++} *		EDTA	0.248	
	2×10^{-8}	"	0.165	33
	5×10^{-8}	"	0.071	64
	5×10^{-7}	"	0.000	100
	1×10^{-3}	"	0.000	100
		Cysteine	0.310	
	1×10^{-1}	"	0.343	0
	1×10^{-3}	"	0.321	0
p-Chloromercuribenzoate†		EDTA	0.350	
	1.5×10^{-7}	"	0.082	77
	3.6×10^{-7}	"	0.044	87
	7.0×10^{-6}	"	0.000	100
		Cysteine	0.370	
	7.0×10^{-6}	"	0.345	0

* 0.01 ml. of cupric or mercuric acetate solution, 0.08 ml. of 0.1 M Tris buffer, pH 7.9, and 0.01 ml. of phosphoribulokinase (300 γ, specific activity 179) were incubated together for 10 minutes at 0°. After this period, 0.02 ml. was removed and assayed spectrophotometrically for kinase activity as described previously. 2 μmoles of activator were added. A control was incubated with the same additions except that water was used in place of metal salt.

† The spectrophotometric assay was employed. The complete system was incubated at 25° before addition of Ru-5-P to start the enzymatic reaction. Cysteine, when present, was added just before Ru-5-P.

sence of activator the final preparation showed little or no activity (Fig. 7). Following prolonged storage at $-16°$, the enzyme was no longer activated by EDTA, although cysteine would restore the activity to its original level. This result suggests that the early activation is due to removal of metal inhibitors, while in the aged enzyme SH groups have become oxidized.

Further evidence for enzymatic SH groups is given by the sensitivity

to heavy metals and p-chloromercuribenzoate (Table V). In these experiments the ADP-phosphoenolpyruvate assay was employed, and it was established that the inhibition (at the concentration of inhibitor employed) was not due to an effect on either phosphopyruvic kinase or lactic dehydrogenase. Hg^{++} showed substantial inhibition at levels of 10^{-8} M, even in the presence of 2×10^{-3} M EDTA. The inhibition by Hg^{++} and by p-chloromercuribenzoate was completely reversed by cysteine. While this result may be simply due to removal of the inhibitor, when taken together with the reactivation studies, it is in accord with the hypothesis that SH groups are essential for the activity of the enzyme.

TABLE VI

Stoichiometry of Phosphoribulokinase Reaction

	Experiment 1	Experiment 2
	μmoles	*μmoles*
Ru-5-P added..	4.00	2.00
ATP disappearance	3.97	2.18
ADP formation	3.44	2.09
Alkali-labile P formed	8.15	3.94

The incubation mixture contained 6.5 μmoles of ATP, 40 μmoles of Tris buffer, pH 7.9, 5 μmoles of Mg^{++}, 2 μmoles of cysteine, 40 units of kinase, specific activity 310, in a total volume of 1.0 ml. After incubation at room temperature for 30 minutes, the pH of the solution was adjusted to 5 0 with 1 N HCl, and the tubes were heated for 1.5 minutes at 100°. Aliquots were removed and assayed for ATP and ADP as described previously. Another aliquot was made 1 N with respect to NaOH, incubated for 25 minutes at room temperature, and the inorganic phosphate determined.

A small but reproducible inhibition was obtained with 5-phosphoribonic acid. Concentrations of 6.6 and 1.33×10^{-3} M 5-phosphoribonic acid caused 33 and 15 per cent inhibition, respectively. R-5-P did not inhibit the esterification of Ru-5-P.

Stoichiometry of Reaction

In the presence of an excess of ATP and relatively large amounts of phosphoribulokinase, Ru-5-P was completely converted to RuDP; an equivalent quantity of ATP was consumed, with the concomitant formation of an equal amount of ADP (Table VI). The absence of Ru-5-P in the final incubation mixture was confirmed by ion exchange chromatography.[9] This balance is in accord with Equation 2. In the succeeding

[9] This experiment was carried out by Dr. P. K. Stumpf.

paper (24) evidence will be presented for the stoichiometric formation of RuDP based on its conversion to 2 moles of phosphoglyceric acid.

DISCUSSION

The presence of a very active phosphoribulokinase in spinach leaves is in accord with the important rôle of RuDP in photosynthesis (2, 3, 25). The crude extract prepared from 1 gm. of leaf tissue will catalyze the formation of approximately 5 μmoles of RuDP from Ru-5-P per minute, while the phosphoriboisomerase activity corresponds to about 200 μmoles of pentose phosphate per minute.

With the aid of these two enzyme preparations, RuDP can now be prepared in substantial amounts. The isolation procedure and proof of structure of the product are reported in the following paper (24).

Both phosphoribulokinase and phosphoriboisomerase exhibit high specificity with respect to substrates. Neither will react with xylulose 5-phosphate, fructose 6-phosphate, or S-7-P. In the case of the former enzyme, the data also indicate that other nucleotides will not substitute for ATP.

SUMMARY

Ribulose diphosphate is formed from ribose 5-phosphate by the action of ATP and two enzymes, phosphoriboisomerase and phosphoribulokinase. These enzymes have been separated and purified from spinach extracts.

The new enzyme, phosphoribulokinase, is specific for ribulose 5-phosphate and ATP. It is activated by divalent metal ions, particularly Mg^{++}, and is inhibited by sulfhydryl-binding agents.

In the presence of excess ATP, ribulose 5-phosphate is completely utilized, and equivalent quantities of ADP and ribulose diphosphate are formed.

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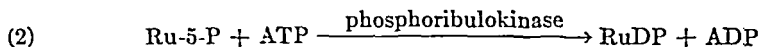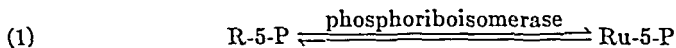25. Calvin, M., *Federation Proc.*, **13**, 697 (1954).

THE ENZYMATIC SYNTHESIS AND PROPERTIES OF
RIBULOSE 1,5-DIPHOSPHATE

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In the preceding paper (1) the rôle of ribulose diphosphate[1] in photosynthetic CO_2 fixation has been reviewed and evidence presented for the participation of the following reactions in its formation:

(1) $$\text{R-5-P} \xrightleftharpoons{\text{phosphoriboisomerase}} \text{Ru-5-P}$$

(2) $$\text{Ru-5-P} + \text{ATP} \xrightarrow{\text{phosphoribulokinase}} \text{RuDP} + \text{ADP}$$

These enzymatic reactions have been utilized for the preparation of RuDP. In the present paper the isolation procedure and proof of structure of RuDP are described, together with some of the properties of this compound. The availability of substrate quantities of RuDP has permitted a direct study of its reaction with CO_2 and the purification of the carboxylation enzyme.[2] This work will be reported in a publication to follow. With purified preparations from spinach, the reaction

(3) $$\text{RuDP} + \text{CO}_2 \xrightarrow{\text{carboxylation enzyme}} \text{2PGA}$$
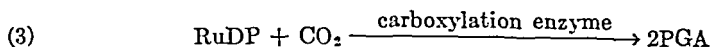
can be employed for the determination of RuDP.

Methods

Unless otherwise specified, the methods and materials employed were as described in the preceding paper.

Ribose 5-phosphate, uniformly labeled with C^{14}, was obtained from the Schwarz Laboratories. Myokinase was prepared by the procedure of Colowick and Kalckar (3) through the ammonium sulfate stage. Perio-

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† Fellow of The National Foundation for Infantile Paralysis.

[1] The following abbreviations are used: RuDP, ribulose diphosphate; Ru-5-P, ribulose 5-phosphate; R-5-P, ribose 5-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PGA, 3-phosphoglycerate.

[2] Calvin *et al.* (2) have suggested the name "carboxydismutase" for this enzyme; in view of its importance in CO_2 fixation the name "carboxylation enzyme" is preferred.

date consumption was measured spectrophotometrically at 265 mμ. This wave-length was employed rather than the maximum at 222 mμ (4), in order to permit the use of higher concentrations of reactants, which greatly increased the rate of the reaction. Formaldehyde was determined by the method of MacFadyen (5). Glycerol was employed as the standard for both measurements; in the chromotropic acid test the absorption at 570 mμ was in agreement with the value given by MacFadyen for equivalent quantities of formaldehyde.

Two preparations of phosphoribulokinase were employed for the synthesis of RuDP. For the later runs the purified enzyme (1) was employed in conjunction with purified phosphoriboisomerase. For the earlier chromatographic procedures (6), a less purified enzyme preparation which contained both isomerase and kinase was found to be satisfactory. For this preparation 60 gm. of spinach leaves were blended at 0° for 3 minutes with 240 ml. of water. The suspension was filtered through Schleicher and Schuell No. 588 filter paper. The filtrate was brought to pH 7.0 with 2 N ammonium hydroxide and centrifuged at high speed (International PR-1, 12,000 × g). The clear supernatant solution (200 ml.) was treated with 51.6 gm. of ammonium sulfate, centrifuged, and the precipitate discarded. The supernatant solution was treated with 35.6 gm. of ammonium sulfate and the precipitate collected and dissolved in 20 ml. of 0.05 M phosphate buffer, pH 7.4. This solution was refractionated with ammonium sulfate as follows. The ammonium sulfate content, tested with a conductivity meter, was found to be 0.14 saturated. 25 ml. of solution were treated with 3.88 gm. of ammonium sulfate and centrifuged. The supernatant solution (23 ml.) was treated with 1.2 gm. of ammonium sulfate and the precipitate collected and dissolved in 5 ml. of 0.05 M phosphate buffer, pH 7.4. 5.0 ml. of this solution (200 mg. of protein) were diluted with 100 ml. of water, and the enzyme was adsorbed with 20 ml. of calcium phosphate gel (7) containing 14 mg. per ml. (dry weight at 105°). The gel was collected by centrifugation and the enzyme eluted with 20 ml. of 0.15 M pyrophosphate buffer, pH 7.6. To concentrate the activity, it was precipitated with 7.6 gm. of ammonium sulfate and dissolved in 2 ml. of 0.05 M phosphate buffer, pH 7.4. For this preparation the manometric assay method of Colowick and Kalckar (3) was employed. The main chamber contained 50 μmoles of sodium bicarbonate, 10 μmoles of MgCl$_2$, 20 μmoles of R-5-P, 0.1 μmole of glutathione, in a total volume of 1.5 ml., and the side arm contained 4 μmoles of ATP in 0.1 ml. The gas phase was 5 per cent CO$_2$ in N$_2$. The temperature was 34°. When this preparation is employed, it is designated as "crude phosphoribulokinase" and the units represent micromoles of CO$_2$ produced in 10 minutes. In all other instances the purified preparations (1) were used.

Preparation of RuDP

Chromatographic Method—In the earlier preparations RuDP was prepared by a large scale adaptation of the manometric assay procedure described in the preceding section (Fig. 1). The solution was placed directly on the Dowex 1 (Cl⁻) column and eluted with an HCl-KCl mixture, with gradient elution. In the chromatogram shown in Fig. 1, RuDP appeared
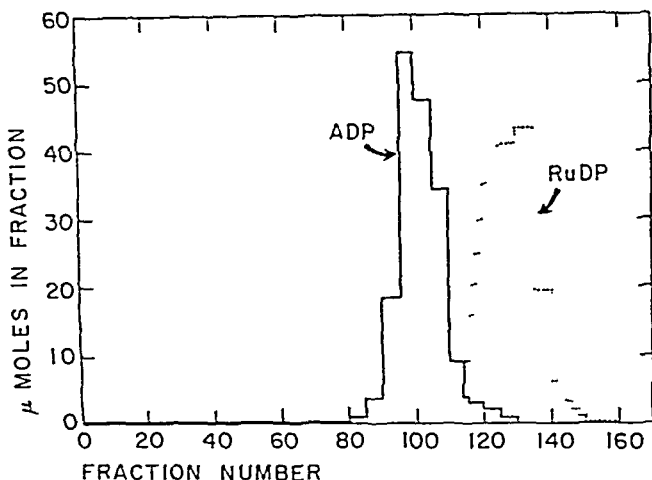

FIG. 1. Chromatographic preparation of RuDP. In this experiment the formation of RuDP from R-5-P and ATP was followed manometrically. The reaction mixture contained 2800 μmoles of R-5-P, 1120 μmoles of ATP, 15 μmoles of ethylenediaminetetraacetic acid, 1170 μmoles of $MgCl_2$ in 168 ml. of 0 05 M $NaHCO_3$ buffer, pH 7.4. The gas phase was 5 per cent CO_2 in N_2. To initiate the reaction, 7 ml of crude phosphoribulokinase (450 units, specific activity 3.5) were tipped in from the side arm. After 30 minutes at 34°, 1000 μmoles of acid were produced. The reaction mixture was placed on a Dowex 1 (Cl⁻) column (7 cm. × 23 cm , 10 per cent cross-linked) and washed with 100 ml. of water. For the gradient elution the mixing chamber contained 1 liter of water and the reservoir 0 2 M KCl in 0 015 M HCl Elution was at a rate of 2 ml. per minute, and the volume of each fraction was 22 5 ml. ADP was calculated from the optical density at 260 mμ; RuDP was estimated from the absorption at 670 mμ in the orcinol reaction

immediately after ADP, although in smaller scale runs, with a relatively larger mixing chamber, better separation was obtained. Fractions 115 to 141 were pooled, concentrated to one-third the original volume, and adjusted to pH 6.3 with NaOH. RuDP was precipitated with 3 ml. of saturated barium acetate, followed by 3 volumes of ethanol. The precipitate was collected by centrifugation, washed with 80 per cent ethanol, and dried *in vacuo* over NaOH and P_2O_5 The dry product weighed 450 mg. It was essentially free of Ru-5-P, R-5-P, and inorganic phosphate and contained only traces of adenine nucleotides. On the basis of organic phos-

phate, it was 77 per cent pure; however, it contained very little inorganic phosphate (less than 3 per cent), and RuDP accounted for all of the organic phosphate. The analytical data are summarized in Table I.

Charcoal Method—For routine large scale preparations it was found more convenient to convert R-5-P completely to RuDP with an excess of ATP and remove residual nucleotides by treatment with charcoal. The progress of the reaction was followed by the quantity of NaOH required to neutralize the acid produced (Fig. 2). When there was no further production of acid, the reaction was stopped by the addition of 0.1 volume of 50 per cent trichloroacetic acid and the nucleotides removed by adsorption on charcoal as described by Crane and Lipmann (8). In the experiment

TABLE I

Analysis of Ribulose Diphosphate Preparations

Analysis	Chromatographed Preparation VI	Charcoal preparation No.			
		IX	X	XI	XII
	*μmoles**	*μmoles**	*μmoles**	*μmoles**	*μmoles**
Organic P..................	266	232	229	258	280
Inorganic P...............	9	105	83	76	52
Ribulose (orcinol)..........	127	114	111	119	115
Ratio, $\dfrac{\text{organic P}}{\text{ribulose}}$..........	2.09	2.03	2.06	2.17	2.43
Purity,† %...............	74	67	65	69	67

* Calculated for 100 mg. of Ba salt (mol. wt. 583).

† Based on the ribulose content.

shown in Fig. 2, 30 gm. of acid-washed Norit A were added and the mixture centrifuged. The charcoal was washed three times with 100 ml. volumes of water, and the combined supernatant solution and washings were adjusted to pH 6.5 with 2.1 ml. of saturated NaOH and 15 ml. of saturated $Ba(OH)_2$. RuDP was precipitated by the addition of an equal volume of ethanol and the precipitate washed with 80 per cent ethanol and dried *in vacuo*. The dry product weighed 540 mg. It was free of R-5-P, ADP, and ATP, but contained appreciable quantities of inorganic phosphate (Table I). Preparations obtained by this method were 60 to 70 per cent pure; however, the bulk of the impurity (15 to 30 per cent) consisted of barium phosphate.

The quantities of ATP and charcoal required in this procedure could be reduced to half by the use of myokinase. In a single preparation carried out with this addition, a product 50 per cent pure was obtained.

Conversion to PGA—An independent assay of the RuDP preparation

was provided by its conversion to PGA in the presence of purified preparations of the carboxylation enzyme (Table II). PGA was determined spectrophotometrically after conversion to phosphoenolpyruvate. The de-
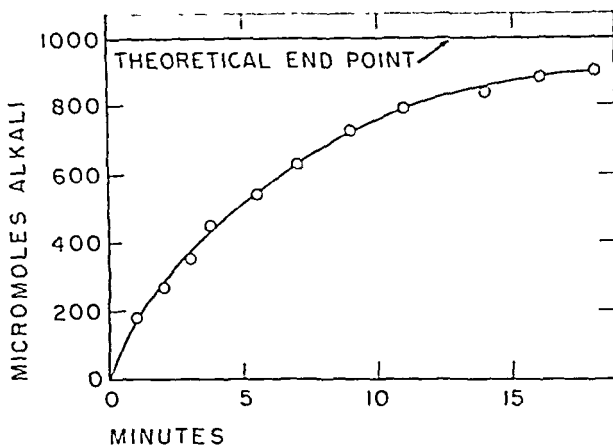

FIG. 2. Alkali consumption in the formation of RuDP. The reaction mixture contained 1000 μmoles of R-5-P, 1130 μmoles of ATP, 2 mg. of phosphopentoisomerase (5000 units), 9.0 mg. of phosphoribulokinase (3850 units), 0.01 M MgCl$_2$, and 0.001 M cysteine, in a total volume of 105 ml. The pH was maintained at 7.9 by the addition of 1 N NaOH, indicated in the figure as micromoles of alkali.

TABLE II

RuDP Assay with Carboxylation Enzyme

Incubation was for 15 minutes at 25° in 0.51 ml. of 0.05 M NaHCO$_3$ saturated with 5 per cent CO$_2$ in N$_2$, containing 0.01 M MgCl$_2$, and an excess (0.05 mg.) of carboxylation enzyme. The reaction mixture was acidified with 0.03 ml. of 1 N HCl heated at 100° for 1 minute and cooled. An aliquot (0.03 ml.) was removed and analyzed for PGA.

Assay	μmoles
PGA formed...................................	0.377
RuDP added*..................................	0.196
PGA:RuDP.....................................	1.93

* Orcinol assay.

tails will be reported in a forthcoming publication. In this experiment the expected quantity of PGA was formed from RuDP, confirming the estimates of purity based on the orcinol and organic phosphorus assays.

One of these enzymatic RuDP preparations has been found by Calvin and his coworkers[3] to be converted to PGA with their algal extracts and to

[3] Personal communication.

correspond with RuDP isolated by chromatography from photosynthesizing plants (9).

Structure of Ribulose Diphosphate

Identification of Ribulose—In previous studies, a preliminary identification of the sugar as ribulose was based on its behavior in paper chromatography (6) and color reactions. In the orcinol reaction the product, either before or after dephosphorylation, gave an absorption spectrum identical

TABLE III

Ribulose o-Nitrophenylhydrazones

300 mg. of barium RuDP (Preparation X, 354 μmoles) were dissolved in 2 ml. of 0.75 N HCl. The barium was removed with 500 mg. of dry Dowex 50 (H+). The combined supernatant solution and resin washings, containing 298 μmoles of pentose, were adjusted to pH 5 and diluted to 10 ml. The solution was treated with 2500 units of potato phosphatase and incubated at 25° for 70 minutes, during which time 595 μmoles of inorganic P were formed. The solution was deionized with a mixed bed column of Duolite A-4 (free base) and IR-120 (H+) (25 ml. of each). The effluent, containing 270 μmoles of pentose, was lyophilized and the residue dissolved in boiling ethanol. The solution was concentrated to 2 ml. and the o-nitrophenylhydrazone prepared according to Glatthaar and Reichstein (10). The yield was 45 mg. (158 μmoles, 57 per cent).

Derivative	M.p.*	Specific rotation, $[\alpha]_D^{20}$†
	°C.	*degrees*
(1) From RuDP......................	163–164	−52
(2) Authentic D-ribulose..............	164–165	−53
(3) " L-ribulose..............	164–165	+52
(1) and (2) mixed....................	163–164	

* Determined with the Fisher-Johns hot stage apparatus.
† c = 0.31 in methanol.

with that produced by authentic ribulose (10), having maxima at 540 and 670 mμ. The ratio $D_{540}:D_{670}$ in this test varied from 0.71 to 0.97, compared to a ratio of 0.80 to 0.90 for authentic ribulose. The intensity of the absorption maximum at 670 mμ was 50 per cent of that given by the aldopentoses (11). In the cysteine-carbazole test the rate of color development was characteristic; the absorption maximum at 540 mμ was reached in 15 to 20 minutes (12), compared with 2 hours for xylulose (13). The identification of ribulose has now been confirmed by the isolation of the o-nitrophenylhydrazone (Table III). The derivative prepared from RuDP had the same melting point and optical rotation as the authentic sample; it was thus established that the sugar had the D configuration. The crys-

tals of the enzymatic and authentic derivatives were identical in microscopic appearance and yielded identical x-ray diffraction patterns.[1]

In order to test for the presence (14) of small quantities of 3-ketopentose, RuDP, uniformly labeled with C^{14}, was prepared and oxidized with periodate. This treatment would convert to CO_2 the carbonyl carbon atom of any 3-ketopentose esterified in the 1,5 positions (see below). In experiments in which 1 μmole of RuDP (19,000 c.p.m., infinitely thin sample) was oxidized with an excess of periodate, negligible quantities of $C^{14}O_2$ were produced. The radioactivity recovered after addition of carrier CO_2 amounted to 2 c.p.m. above background in one case and 13 c.p.m. above background in another case (counted at infinite thickness). It can therefore be calculated that 3-ketopentose, if present, amounted to less than 3 per cent of the total pentose. In agreement with this result, it was found that the specific color test for 3-ketopentoses (15) was not given by the RuDP preparations, even in high concentration.

Position of Phosphate Groups—The analytical data (Table I) indicated the presence of 2 moles of organic phosphate per mole of ribulose, presumably esterified at positions 1 and 5. This was confirmed by periodate oxidation (Table IV). It was found that 2 moles of periodate were consumed per mole of pentose, and only traces of formaldehyde and carbon dioxide were formed. This result would be given only by ribulose esterified in the 1 and 5 positions; the 4,5 derivative, which would also consume 2 equivalents of periodate, would yield 1 equivalent each of formaldehyde and CO_2.

Other Properties of D-Ribulose 1,5-Diphosphate

Alkali Lability—RuDP was found to be decomposed by alkali at room temperature, with the rapid liberation of inorganic phosphate (Fig. 3). At 25°, the half life of RuDP was 2.8 minutes in 1 N NaOH and 15 minutes in 0.1 N NaOH. The appearance of inorganic phosphate was found to follow first order kinetics with no change in slope, indicating that the two phosphate groups were equally labile.

Acid Lability—In 1 N H_2SO_4 at 100° the hydrolysis of RuDP was a first order reaction with no change in slope, again indicating equal lability of the two phosphate groups (Fig. 3). Under these conditions half of the total phosphate was liberated in about 20 minutes, compared with about 40 minutes for Ru-5-P (11). It would appear that the presence of the 1-phosphate group renders the 5 position linkage more labile to acid.

Enzymatic Properties—RuDP is very slowly cleaved by crystalline aldolase (16); the rate is less than one-fiftieth of that obtained with fructose diphosphate. It is not attacked by purified spinach transketolase (17).

[1] We are indebted to Mr. Wm. C. White of the National Cancer Institute for carrying out the x-ray diffraction studies.

It is rapidly hydrolyzed by a phosphatase present in spinach leaves, with the production of 1 equivalent of inorganic phosphate. On purification this activity appears to parallel a fructose diphosphatase (18) which is

<center>TABLE IV</center>

<center>*Periodate Oxidation of RuDP*</center>

The reaction mixtures (1.05 ml.) contained 10^{-3} M H_5IO_6 in 0.1 M acetate buffer, pH 4.5. Density readings were made at 265 mμ in a cell of 1.0 cm. light path. After the initial readings the test compounds were added and the absorption followed until no further changes occurred. Reagent grade glycerol was used as a standard and a sample of crystalline sedoheptulosan (kindly provided by Dr. N. K. Richtmyer of this Institute) was run for comparison. When the reaction was complete (about 45 minutes with RuDP), 0.05 ml. of 0.01 M sodium arsenite was added, and 0.5 ml. aliquots were removed for formaldehyde assay (5).

	RuDP, Preparation IX	RuDP, Preparation X	Glycerol	Sedoheptulosan
Substrate added, $\mu mole$................	0.231*	0.214*	0.160	0.200
ΔD (265 mμ).........................	0.228	0.223	0.160	0.217
" (670 ")†.........................	0.037	0.010	0.266	0.017
Periodate consumed, $\mu mole$‡...........	0.456	0.446	(0.320)§	0.434
HCHO formed, $\mu mole$.................	0.04	0.01		0.02
$\dfrac{\text{Periodate}}{\text{Pentose}}$....................	1.97	2.08		2.17
$\dfrac{\text{HCHO}}{\text{Pentose}}$....................	0.17	0.05		0.10

    * By the orcinol method.

    † In the chromotropic acid method for HCHO.

    ‡ Based on the density change produced by the glycerol standard.

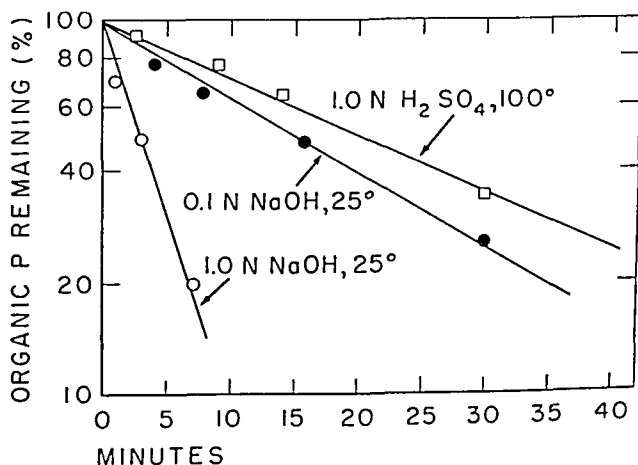   § Theory for 0.160 μmole of glycerol.

FIG. 3. Hydrolysis of RuDP under acid and alkaline conditions

present in plant extracts.[5] The product of RuDP hydrolysis by this enzyme has not yet been identified.

DISCUSSION

The compound detected by Benson (9) on paper chromatograms of extracts of photosynthesizing cells has now been prepared by enzymatic methods and its identity as D-ribulose 1,5-diphosphate firmly established. Little doubt remains as to the function of this compound as the primary CO_2 acceptor in photosynthesis, thus ending the long search for the so called "C_2 acceptor." Work in progress in this laboratory and in others (2, 14, 19, 20) has served to establish the important rôle of the phosphate esters of ribulose in photosynthesis. While no function for RuDP has yet been found in animal tissues and heterotrophic microorganisms, it may be surmised that this compound, with its highly reactive carbonyl group, is employed for other biosynthetic mechanisms.

SUMMARY

Methods for the preparation of ribulose diphosphate are described, based on the enzymatic synthesis of this compound from pentose phosphate.

The pentose has been identified as D-ribulose by conversion to the o-nitrophenylhydrazone. 2 equivalents of phosphate are present, and on the basis of periodate oxidation it has been established that these are esterified in the 1 and 5 positions. Both phosphate groups are equally labile to acid or alkaline hydrolysis. The product yields 2 equivalents of 3-phosphoglyceric acid in the presence of the carboxylation enzyme.

Spinach leaves contain a phosphatase which hydrolyzes one of the two phosphate groups of ribulose diphosphate.

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THE ENZYMATIC FORMATION OF PHOSPHOGLYCERIC ACID FROM RIBULOSE DIPHOSPHATE AND CARBON DIOXIDE

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Calvin and his coworkers have shown that CO_2 fixed in photosynthesis is first detected in the carboxyl group of phosphoglyceric acid and have proposed that this compound is produced in the primary carboxylation reaction (1). Fager (2) has reported the incorporation of CO_2 into PGA[1] in cell-free plant extracts, which was stimulated by an unidentified phosphate ester present in the extracts. Evidence for the utilization of pentose esters for this process has been reviewed (3). It is now apparent that the immediate precursor of PGA is ribulose diphosphate (4–7), which arises from ribulose 5-phosphate and ATP in the presence of phosphoribulokinase (3). In an accompanying publication (8) the enzymatic synthesis, isolation, and proof of structure of this intermediate (RuDP) have been discussed.

The present communication is concerned with an enzyme from spinach which catalyzes the formation of PGA from RuDP and CO_2 as shown in equation (1).[2] This reaction does not appear to be reversible at neutral
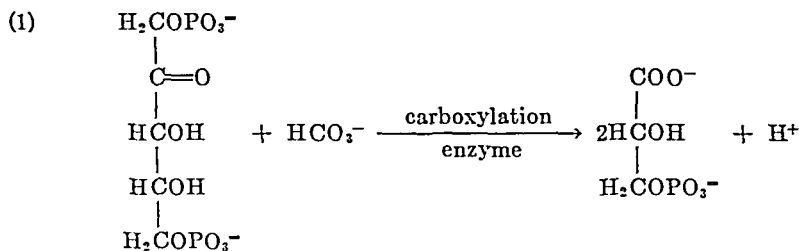
(1)

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[1] The following abbreviations are used: PGA, phosphoglyceric acid; RuDP, ribulose diphosphate; Ru-5-P, ribulose 5-phosphate; R-5-P, ribose 5-phosphate; G-6-P, glucose 6-phosphate; HDP, hexose diphosphate; DPN, diphosphopyridine nucleotide; DPNH, dihydrodiphosphopyridine nucleotide; ATP, adenosine triphosphate; ADP, adenosine diphosphate; GSH, glutathione; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TEA, triethanolamine.

[2] Under the conditions of most experiments (pH 7.7 and 5 per cent CO_2) bicarbonate ion is the principal species. This is not intended to imply that it is the primary reactant.

pH. The product has been identified as PGA by ion exchange chromatography, oxidation with sodium periodate after dephosphorylation, and by enzymatic assay methods.

Simultaneously with the work reported here, the presence of the carboxylation reaction in spinach leaves was reported by Racker (9) and by Jakoby, Brummond, and Ochoa (10).

Methods

Materials—The following substances were commercial preparations: ATP (crystalline) and ADP, Sigma Chemical Company; GSH, Nutritional Biochemicals Corporation; PGA and HDP (barium salts), Schwarz Laboratories; sodium metaperiodate, G. Frederick Smith Chemical Company; Dowex 1, The Dow Chemical Company. DPN was prepared by the method of Kornberg and Pricer (11), and DPNH by the method of Ohlmeyer (12). Ru-5-P (13), RuDP (8), and sedoheptulose 7-phosphate (14) were prepared as described previously. Glycolaldehyde phosphate (diose phosphate) was prepared by the method of Fleury and Courtois (15). 1-Pyrophosphoryl ribose 5-phosphate (16) was a gift of Dr. A. Kornberg of Washington University. Uniformly labeled C^{14}-HDP was purchased from the Nuclear Instrument and Chemical Corporation (Chicago). Uniformly labeled C^{14}-R-5-P, used to prepare C^{14}-RuDP, was obtained from the Schwarz Laboratories. R-5-P-1-C^{14} was prepared as described previously (17). BaC^{14}O$_3$ was provided by the Atomic Energy Commission.

Enzyme Preparations—Potato phosphatase was purified by the method of Kornberg and Pricer.[3] Crystalline aldolase (18) and glyceraldehyde 3-phosphate dehydrogenase (19) were isolated from rabbit muscle. Phosphoglyceryl kinase was prepared from peas (20).

A crude rabbit muscle fraction employed for the determination of PGA was prepared as follows: The extract obtained in the procedure for aldolase (18) was adjusted to pH 7.7 and treated with 0.5 volume of ammonium sulfate solution (saturated at 0°). Concentrated ammonium hydroxide was added slowly to bring the pH to 7.5. The suspension was centrifuged, and the supernatant solution was treated with 30 gm. of ammonium sulfate per 100 ml. The bulky precipitate was collected by centrifugation, suspended in a small volume of water (about 10 ml. per 100 ml. of original extract), and dialyzed overnight against cold, flowing, distilled water. Insoluble material was removed by centrifugation. The solution was stored at −16° and retained its activity over a period of several months; a precipitate which developed during this time was removed by centrifugation. This solution provides all of the enzymes necessary for the reactions shown in Diagram 1.

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[3] Unpublished procedure.

Determinations—Protein was determined by the method of Sutherland *et al.* (21). Phosphate analyses were carried out by the procedure of Fiske and Subbarow (22). Absorption measurements were made with a Beckman DU spectrophotometer. Electrophoresis experiments were carried out at 25° with the Aminco-Stern apparatus and ultracentrifugation experiments with the Spinco model E centrifuge. Radioactivity measurements were made with a gas flow counter.

Isolation and Degradation of PGA—For the isolation of PGA, Dowex 1 Cl⁻ columns (3.8 sq. cm. × 23 cm.) were employed. Elution was by the gradient technique (23) at a rate of 0.5 ml. per minute, with 750 ml. of water in the mixing chamber and 0.1 N HCl in the reservoir. Under these conditions PGA was eluted in about 6.5 column volumes, well separated from sedoheptulose 7-phosphate, glucose 6-phosphate, ribose 5-phosphate, and 6-phosphogluconate, which appear in about 3.5, 4.0, 4.5, and 5.0 column volumes, respectively. Fructose diphosphate was not eluted, even after 8 column volumes of eluate had been collected. Fractions containing PGA (determined by C^{14}, total P, or enzymatic assay) were pooled and taken to dryness *in vacuo*, and the residue was dissolved in water. Barium acetate was added, and the barium salt of PGA was precipitated at pH 7 with an equal volume of ethanol. The precipitate was washed with 50 per cent ethanol, extracted with 0.1 N HCl, and reprecipitated by adjusting to pH 7 with NaOH. This procedure was repeated to constant specific activity. To decompose the barium salt the precipitate was suspended in water and treated with a small excess of K_2SO_4.

For the degradation of PGA, solutions of potassium salt at pH 5.0 were hydrolyzed with potato phosphatase, and the free glyceric acid formed was oxidized with sodium periodate as described by Sakami (24) for the degradation of serine.

Results

Carboxylation Enzyme

Assay—The assay for the carboxylation enzyme was based on the rate of formation of PGA from RuDP and CO_2 and was carried out in two steps. In the first step RuDP and CO_2 were incubated with the carboxylation enzyme in a reaction mixture which contained 25 μmoles of $NaHCO_3$, 5 μmoles of $MgCl_2$, 3 μmoles of GSH, 0.03 μmole of EDTA, and 0.2 μmole of RuDP, in a total volume of 0.52 ml. The solution was saturated with 5 per cent CO_2 in N_2, treated with 0.02 ml. of enzyme solution (appropriately diluted), and incubated at 25° for 10 minutes. The reaction was stopped by the addition of 0.03 ml. of 1.0 N HCl, and the solution was heated in a boiling water bath for 1 minute.

In the second step an aliquot (usually 0.1 ml.) was taken for PGA assay (Diagram 1).   The solution contained 50 μmoles of Tris buffer, pH 7.7, 10 μmoles of $MgCl_2$, 0.07 μmole of DPNH, 0.05 ml. of dialyzed rabbit muscle preparation, and the sample to be assayed, in a quartz cuvette with a total volume of 1.0 ml. and light path of 1.0 cm.   The optical density was measured at 340 mμ, following which 0.4 μmole of ADP (0.02 ml.) was added.   Usually 15 to 20 minutes were required for the reaction to come to completion.   The quantity of DPNH oxidized, calculated from the change in absorption at 340 mμ ($\epsilon = 6.22 \times 10^3$), was equal to the amount

3-Phosphoglycerate

| [phosphoglycerate mutase]
↓

2-Phosphoglycerate

| [enolase]
↓

Phosphoenolpyruvate

ATP ← ADP | [phosphoenolpyruvate kinase]
↓

Pyruvate

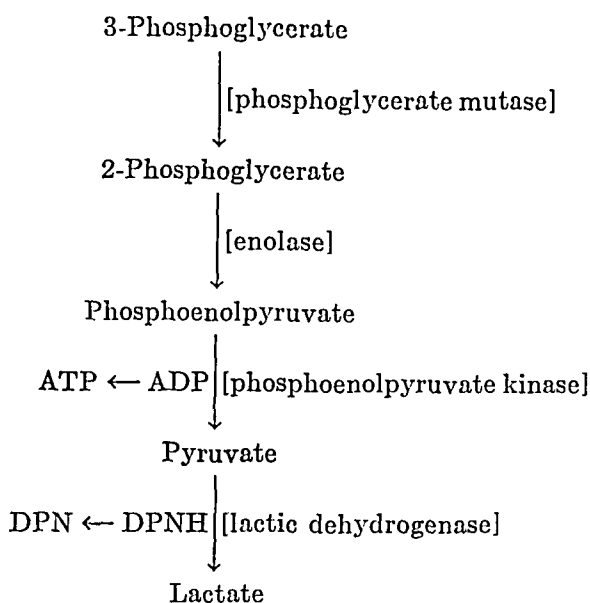DPN ← DPNH | [lactic dehydrogenase]
↓

Lactate

DIAGRAM 1. The enzymatic assay for phosphoglyceric acid.   The experimental conditions were as described in the text (second step).   No oxidation of DPNH was observed until ADP was added.

of PGA added in the aliquot.   Identical values for PGA were obtained when aliquots were assayed according to Bücher (25) by conversion to 1,3-diphosphoglyceric acid with phosphoglyceryl kinase and ATP and reduction to glyceraldehyde 3-phosphate with DPNH and glyceraldehyde 3-phosphate dehydrogenase.   A unit of carboxylation enzyme was defined as the amount which would form 1.0 μmole of PGA in 10 minutes under the conditions of incubation with RuDP.   Specific activity represents the number of units per mg. of protein.   The proportionality of rate to enzyme concentration is illustrated in Fig. 1.

Purification—Fresh spinach leaves from the local market were washed with cold tap water and the stems discarded.   600 gm. of leaf material were homogenized at 0° in a large Waring blendor with 2000 ml. of solution containing 10^{-4} M EDTA and 10^{-2} M K_2HPO_4 adjusted to pH 7.4.

After 3 minutes at top blendor speed, the homogenized suspension was filtered through Schleicher and Schuell No. 5SS fluted filter paper. The opaque dark green filtrate was adjusted to pH 7 with 1 N ammonium hydroxide ("crude extract," 2100 ml., Table I). This extract was treated with 475 gm. of ammonium sulfate and centrifuged. The pale green su-
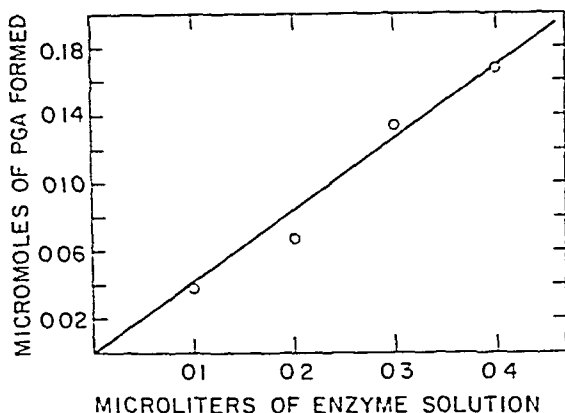

Fig. 1. Rate of PGA formation as a function of the concentration of carboxylation enzyme. The assay was as described in the text.

TABLE I

Purification of Carboxylation Enzyme

	Total units*	Specific activity*
Crude extract	35,300	5.5
Ammonium Sulfate I	28,700	16.7
" " II	21,300	31.6
Heated fraction	21,300†	37.6
Aluminum hydroxide eluate	17,600†	52.0

* As defined in the text.

† The recoveries were corrected for the aliquots taken in these steps.

pernatant solution was treated with 210 gm. of ammonium sulfate, centrifuged, and the precipitate dissolved in 100 ml. of 0.1 M phosphate buffer, pH 7.4, containing 5×10^{-5} M EDTA (Ammonium Sulfate I, 114 ml.).

The solution obtained in the previous step was diluted with an equal volume of cold water. At this point the degree of saturation with respect to ammonium sulfate, determined by conductivity measurement with the Barnstead purity meter, was 0.07. The diluted solution (226 ml.) was treated with 92.5 ml. of a saturated ammonium sulfate solution (saturated at room temperature and adjusted to pH 7.3 with concentrated NH_3).

The suspension was centrifuged, the supernatant solution treated with 26.4 ml. of saturated ammonium sulfate solution, and the precipitate again discarded. To the supernatant solution were added 30.3 ml. of saturated ammonium solution, and the precipitate was collected by centrifugation and dissolved in 30 ml. of 0.1 M phosphate buffer, pH 7.4 (Ammonium Sulfate II, 36 ml.).

A portion of this solution (27 ml.) was heated to 60–63° in approximately 3 minutes, held for 5 minutes at this temperature, and rapidly cooled and centrifuged. The residue was washed with 5 ml. of water and discarded. The supernatant solution and washing were combined ("heated fraction," 28 ml.).

The heated fraction was diluted with 140 ml. of cold water and treated with 196 ml. of aged aluminum hydroxide Cγ gel (12.7 mg. per ml.) (26). The suspension was centrifuged and the enzyme eluted from the gel with 50 ml. of 0.1 M phosphate buffer, pH 7.7 ("aluminum hydroxide eluate," 77 ml.).

Properties of Carboxylation Enzyme

Stability—While the most purified preparations were only moderately stable and lost appreciable activity when stored for several days at −16°, the preparation at the stage of Ammonium Sulfate II exhibited a slow but steady increase in activity at −16°. With some preparations this increase continued for 4 to 6 weeks and amounted to over 100 per cent. Presumably this effect can be attributed to the presence of an unstable inhibitor in the spinach extracts. Owing to this phenomenon, the true capacity of extracts to catalyze the carboxylation reaction could not be evaluated.

The carboxylation enzyme is unstable below pH 6. Because of this instability at low pH, it was important to prepare the extracts in the presence of an alkaline buffer, since unbuffered spinach extracts have an acidic reaction.

Homogeneity—Although the best preparations were purified only about 10-fold compared with spinach extracts, further efforts to increase the specific activity proved unsuccessful. Electrophoresis and ultracentrifugation studies, carried out in collaboration with Dr. W. R. Carroll of this Institute, indicated that the bulk of the protein was associated with a single homogeneous component. In the electrophoresis runs (Fig. 2), a single rapidly moving boundary was present. The mobility at pH 7.7 was greater than that of serum albumin, indicating that the molecule carries a substantial net negative charge at this pH. Patterns nearly identical with that in Fig. 2 were also obtained at pH 7.7 and 8.6, except that at the more alkaline pH the descending boundary showed a tendency to split into two peaks toward the end of the run. This result may be due to the instability of the enzyme under the conditions of the experiment.

The ultracentrifugation pattern had two very minor peaks in addition to the major component (Fig. 3). The sedimentation constant for the major peak was 17 Svedberg units, measured in 0.1 M TEA buffer, pH 6.9,

FIG. 2. Electrophoresis pattern of the purified carboxylation enzyme. The upper curve represents the ascending boundary and the lower curve the descending boundary after 230 minutes at 12.5 ma. The aluminum hydroxide eluate was precipitated with ammonium sulfate, dissolved in buffer, and dialyzed against TEA buffer, pH 6.85, 0.1 ionic strength. The protein concentration after dialysis was 14.6 mg. per ml.

FIG. 3. Ultracentrifugation pattern of the purified carboxylation enzyme. The enzyme preparation described in Fig. 2 was dialyzed overnight against TEA buffer, pH 8.9, at 0.1 ionic strength. The solution was diluted with buffer to a protein concentration of 5 mg. per ml. The progress of ultracentrifugation at $260,000 \times g$ is shown from left to right. With other preparations a very small rapidly moving component was observed; this was absent in the run illustrated.

at a protein concentration of 0.5 per cent. The diffusion constant was found to be 5.5×10^{-7} sq. cm. per second in 0.1 M Tris buffer, pH 6.9, at a protein concentration of 2 per cent. From these data the molecular weight was estimated to be about 300,000. In the ultracentrifuge as well as in the electrophoresis experiments, the enzymatic activity was always associated with the major protein peak.

Substrate Specificity—Purified preparations of the carboxylation enzyme were devoid of phosphoribulokinase, transketolase, or transaldolase. Of a number of possible substrates tested, only RuDP served as an acceptor for carbon dioxide (Table II). Neither ribose 1,5-diphosphate nor ribulose 5-phosphate served as a substrate for the enzyme as measured by PGA formation. Maximal activity was reached at an RuDP concentration of about 5×10^{-4} M; higher concentrations were inhibitory (Fig. 4). From the Lineweaver-Burk plot (27) K_s was estimated to be 2.5×10^{-4} M; the extrapolated value for $V_{max.}$ was nearly twice the observed maximal velocity.

TABLE II

Specificity of Carboxylation Enzyme

Substrate	Counts per min. fixed
None	0
RuDP	9400
Diose phosphate	38
Hexose diphosphate	38
Sedoheptulose diphosphate	0
R-5-P	0
Glucose 6-phosphate	0
Fructose 1-phosphate	0

The incubation mixtures contained 25 μmoles of $NaHCO_3$, 5 μmoles of $MgCl_2$, 3 μmoles of GSH, 0.3 μmole of substrate, 4 μmoles of $K_2C^{14}O_3$ containing 3×10^6 c.p.m., and 6.3 units of carboxylation enzyme in 0.6 ml. The mixtures were gassed with 5 per cent CO_2 in N_2 before addition of enzyme and radioactive carbonate. After 10 minutes at room temperature, 0.03 ml. of 10 N H_2SO_4 was added, and the solution was heated at 100° for 1 minute. After removal of $C^{14}O_2$ from the reaction mixture by sweeping with N_2, an aliquot of the solution was plated, dried, and counted.

Half maximal velocity was obtained at a bicarbonate ion concentration of 1.1×10^{-2} M. At pH 7.7 and 25° the H_2CO_3 concentration in equilibrium with this concentration of bicarbonate ion would be 1.8×10^{-4} M.

Cofactor Requirements—Both Mg^{++} and a sulfhydryl compound or sequestering agent were required for full activity. As shown in Table III, the purified enzyme was completely inactive in the absence of added metal ions; Ni^{++} and Mg^{++} were equally effective activators, while Co^{++}, Mn^{++}, and Fe^{++} were less active. Fe^{+++} was completely inactive. The stimulatory effect of sulfhydryl compounds such as GSH or cysteine varied with the stage of preparation and the age of the enzyme. The particular preparation employed in the experiment described in Table IV was activated 10-fold by the addition of sulfhydryl compounds. EDTA in place of GSH produced similar activation. No other cofactors have been detected.

Microbiological assays failed to reveal the presence of significant quantities of thiamine, riboflavin, nicotinamide, folic acid, or lipoic acid containing cofactors.
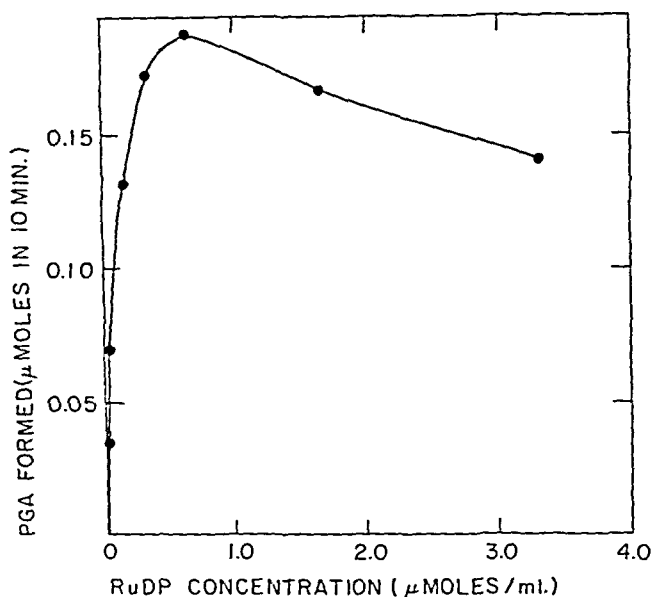

Fig. 4. Effect of RuDP concentration on the rate of PGA formation. The reaction conditions were as described in "Methods."

TABLE III

Metal Activation of Carboxylation Enzyme

Additions	Concentration	PGA formed
	M	μmole per 10 min.
None*		0.007
Mg^{++}	10^{-2}	0.149
Ni^{++}	10^{-2}	0.151
Co^{++}	10^{-2}	0.068
Mn^{++}	10^{-3}	0.025
Fe^{++}	10^{-3}	0.021

* The enzyme assays were carried out as described in "Methods" with 1.75 units of the carboxylation enzyme at the stage of Ammonium Sulfate II. The metal ions were added as the chlorides.

Effect of pH—The carboxylation enzyme was most active at about pH 8; there was little or no activity below pH 6.4 or above 9.2 (Fig. 5). The low activity below pH 7 may be due in part to the instability of the enzyme on the acid side of neutrality.

Effect of Inhibitors—The carboxylation enzyme was inhibited by relatively low concentrations of phosphate or arsenate ions. Thus in 0.03 M and 0.01 M phosphate, the activity was only 10 and 30 per cent of the con-

TABLE IV

Glutathione Stimulation of Carboxylation Enzyme

GSH addition	PGA formed
	μmole per 10 min.
None..	0.010
5 × 10⁻⁴ M....................................	0.038
3 × 10⁻³ "......................................	0.097
7 × 10⁻³ "......................................	0.100

The assay conditions were as described in "Methods." For this experiment a preparation at the stage of Ammonium Sulfate I (10 ml.) was dialyzed overnight against 0.05 M sodium acetate and fractionated at pH 6.3 and 0° with ethanol. The precipitate obtained between 20 and 30 per cent ethanol was dissolved in 10 ml. of water, adjusted to pH 6.3, and fractionated at 0° with methanol. The fraction collected between 19 and 25 per cent methanol was dissolved in 2 ml. of 0.1 M TEA buffer, pH 7.7. 9.3 γ of this preparation were used in each vessel.
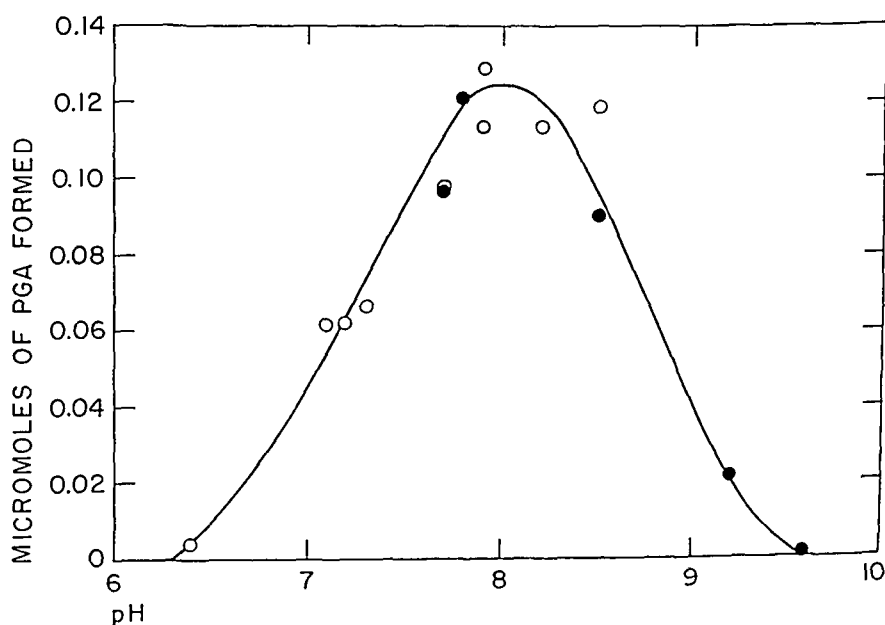

FIG. 5. The effect of pH on reaction velocity. The assay conditions were as described in "Methods" except for the buffer utilized. The following buffers were employed: TEA, pH 6.3 and 7.9; succinate, pH 6.4; bicarbonate, pH 7.2, 7.3, 7.7, 8.5, 9.2, and 9.6; glycylglycine, pH 7.4; Tris, pH 7.8; glycine, pH 7.9, 8.2, and 8.5. In the experiments designated by ●, the gas space was air; in those designated by O, it was 5 per cent CO₂ in N₂. The pH values were determined at the end of the incubation period.

trol value, respectively. Arsenate at 0.02 M gave complete inhibition. HgCl$_2$ at 2 × 10^{-4} M inhibited completely, while p-chloromercuribenzoate at the same concentration inhibited 50 per cent. Arsenite at 10^{-3} M was without effect.

Effect of Temperature—The rate of RuDP carboxylation increased approximately 2-fold for every 10° increase in temperature (Fig. 6). From the slope of the line obtained by plotting the log of the velocity against $1/T$, the activation energy calculated from the Arrhenius equation was 16,900 calories per mole.
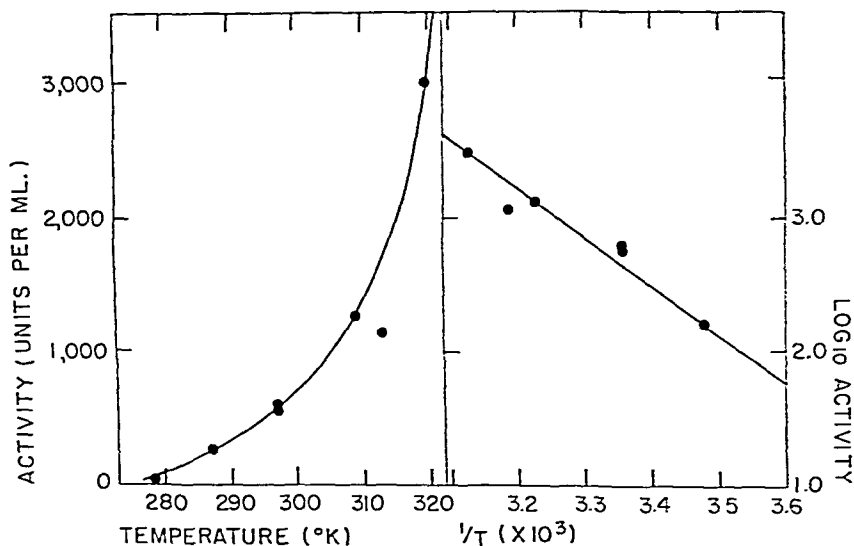

Fig. 6. The effect of temperature on the reaction velocity. The reaction conditions were as described in the text, with an enzyme preparation carried through Ammonium Sulfate II. The specific activity was 30.4 units per mg. The quantities of enzyme used ranged from 1.9 γ at 46.5° to 39 γ at 4°.

Phosphoglyceric Acid Formation

Isotope Experiments—In order to provide information on the mechanism of PGA formation, experiments were performed with labeled CO$_2$ or R-5-P. When C^{14}O$_2$ was employed, RuDP was the other substrate; with R-5-P-1-C^{14}, the CO$_2$ was unlabeled and phosphoriboisomerase (3), phosphoribulokinase (3), and ATP were added, in addition to the carboxylation enzyme. PGA produced from C^{14}O$_2$ and RuDP contained all of the radioactivity in the carboxyl group. With 1-labeled pentose phosphate as the substrate, 99 per cent of the isotope was in the β position (Table V). This result is to be compared with that obtained with a crude spinach extract (28) in which about 25 per cent of the total radioactivity of the PGA

was found in the carboxyl carbon atom. It is now clear that the spread of isotope observed with the crude preparations can be attributed to side reactions rather than to the carboxylation reaction itself.

Stoichiometry—In the presence of excess CO_2, 1 mole of RuDP yields 2 moles of PGA (Fig. 7). This is consistent with the observation of Jakoby, Brummond, and Ochoa (10), who obtained 2 moles of PGA per mole of CO_2 utilized. In agreement with these workers, no evidence could be obtained for the reversibility of the reaction with PGA as the substrate,

TABLE V

Distribution of C^{14} in PGA Formed from Labeled Substrates

Carbon atom	Specific activity	
	With $C^{14}O_2$*	With R-5-P-1-C^{14}†
	c.p.m. per mg. C	*c.p.m. per mg. C*
Carboxyl	9350	19
α	1	48
β	0	4840

* The reaction mixture (0.9 ml.) contained 20 μmoles of TEA buffer, 10 μmoles of NaHCO₃, 0.18 μmole of RuDP, 17 μmoles of K₂C¹⁴O₃ (2 × 10⁶ c.p.m.), 5 μmoles of MgCl₂, and 16 units of carboxylation enzyme. The pH was 7.7. After 40 minutes at 25° the reaction was stopped by the addition of 1.0 ml. of 10 per cent trichloroacetic acid, and CO₂ was removed by sweeping for 5 minutes with N₂. Carrier PGA (300 μmoles) was added. The chromatographic isolation and degradation were as described in "Methods."

† The reaction mixture (2.52 ml.) contained 3 μmoles of R-5-P-1-C¹⁴ (79,000 c.p.m.), 10 μmoles of ATP, 20 μmoles of MgCl₂, 33 μmoles of GSH, 100 μmoles of bicarbonate, 50 units of phosphoribulokinase, 30 units of phosphoriboisomerase, and 60 units of carboxylation enzyme. Before addition of enzymes the solution was gassed with 5 per cent CO₂ in N₂. After 30 minutes the reaction was stopped with 0.06 ml. of 1 N HCl and heated for 1 minute at 100°. Carrier PGA (110 μmoles) was added. Isolation and degradation procedures were as described in "Methods."

either by $C^{14}O_2$ incorporation or pentose formation. Preliminary determinations of ΔH for reaction (1) (see the introduction), carried out in collaboration with Dr. T. H. Benzinger and Dr. C. Kitzinger of the Naval Medical Research Institute,[4] indicate a value of approximately −5000 calories per mole. If ΔF and ΔH are of similar magnitude, assuming the entropy change to be small, this result is consistent with the observed irreversibility. Because of the high affinity of the carboxylation enzyme for RuDP, the reaction is suitable for the quantitative determination of RuDP. The application of this method is illustrated in the accompanying publication (8).

4 A detailed account of these studies will be published elsewhere.

Intermediates—It has been suggested that a 3-ketopentose diphosphate may act as the CO_2 acceptor in the carboxylation reaction (9). No evidence for such an intermediate has been obtained. As discussed elsewhere (8), oxidation of a 3-ketopentose diphosphate with periodate would yield CO_2 from the carbonyl carbon atom. Uniformly labeled C^{14}-RuDP (1 μmole, 19,000 c.p.m., counted at infinite thinness) was incubated with 2.5 units of the carboxylation enzyme for 5 minutes in the absence of CO_2, following which it was treated with an excess of periodate. The radioactivity present as CO_2, isolated after the addition of 120 μmoles of carrier bicarbonate, amounted to 2 ± 1 c.p.m. (counted as $BaCO_3$ at infinite
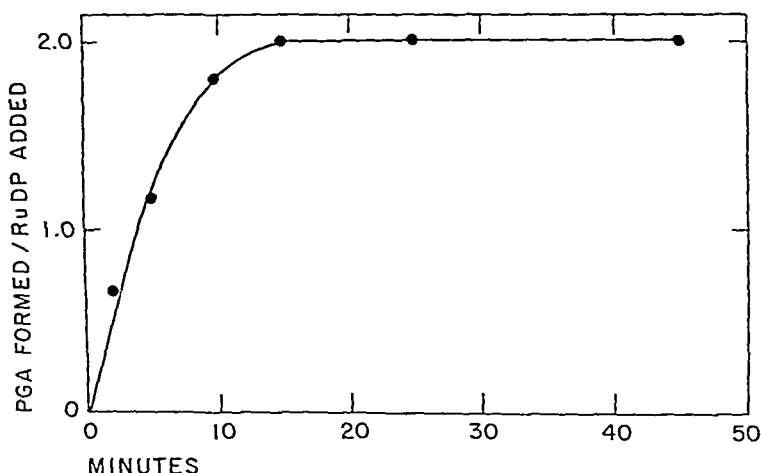

Fig. 7. The conversion of RuDP to PGA. The reaction conditions and PGA assay were as described in the text, except that 0.1 μmole of RuDP was present. The carboxylation enzyme was Ammonium Sulfate II, specific activity 29.7. In each experiment, 0.17 unit of enzyme was added.

thickness). Corrected to counts at infinite thinness, this accounted for less than 1 per cent of the total radioactivity added. Thus less than 1 per cent of a 3-keto derivative was present. Essentially the same result was obtained with RuDP before incubation with the carboxylation enzyme (8). Analysis of similar incubation mixtures for 3-ketopentose by the specific color test of Ashwell and Hickman (29) was also negative.

Radioactive triose phosphate, generated from C^{14}-HDP with aldolase, does not appear in PGA formed from RuDP. In this experiment RuDP (1.3 μmoles) was converted quantitatively to PGA by the carboxylation enzyme in the presence of 1.68 μmoles of C^{14}-HDP, containing 9000 c.p.m., and an excess of aldolase. Carrier PGA (50 μmoles) was added to the incubation mixture, which was then made 1 N with respect to HCl and heated at 100° for 1 hour to destroy the labile phosphate esters. The mixture was

then neutralized and the PGA isolated by ion exchange chromatography as described in "Methods." No radioactivity could be detected in the recovered PGA, indicating that free triose phosphate was not formed during the carboxylation reaction.

When 2,6-dichlorophenolindophenol (30) was added to an incubation mixture containing RuDP, EDTA, and the carboxylation enzyme either in the presence or absence of CO_2, no reduction of the dye was noted, indicating that a free enediol derivative does not accumulate. Under similar conditions the addition of ascorbic acid or GSH to the reaction mixture resulted in immediate reduction of the dye.

DISCUSSION

The demonstration of phosphoribulokinase (7) and of the carboxylation enzyme in spinach leaf extracts provides direct experimental support for the concept that RuDP acts as the primary carbon dioxide acceptor in photosynthesis. Assuming a molecular weight of approximately 300,000, the turnover number at 25° was calculated to be approximately 1500 moles of PGA produced per mole of enzyme per minute. This relatively low turnover rate is compensated for by a high concentration of the enzyme, which appears to constitute 5 to 10 per cent of the soluble protein of spinach leaf. Further evidence will be required to confirm the homogeneity of the purified preparations; until such evidence is available, the turnover number and quantity of enzyme in spinach tissue must be regarded as tentative values. Other sources of enzyme, such as rye grass cuttings, were found to yield preparations very similar to those obtained from spinach.

Several lines of evidence are available for the identity of the reaction product, including (a) chromatography with authentic PGA on ion exchange resins, (b) enzymatic conversion to phosphoenolpyruvate and subsequent reduction to lactate, (c) enzymatic phosphorylation to 1,3-diphosphoglyceric acid and reduction to glyceraldehyde 3-phosphate (25), (d) degradation with periodate following enzymatic dephosphorylation. In addition the stability of the reaction product to acid hydrolysis was consistent with that of PGA. In the chromatographic isolation, carrier PGA was eluted in constant proportion to the radioactivity. Although neither of the enzymatic assays is completely specific, each yielded quantitatively identical results, leaving little doubt that the product was indeed PGA. In the reaction with periodate only serine and glyceric acid would yield the products obtained; the absence of a ninhydrin reaction excluded the former.

With respect to the mechanism of the carboxylation reaction, no final conclusion can be drawn. The results obtained here neither support nor deny the enediol mechanism proposed by Calvin (31), although the re-

quirement of the carboxylation reaction for a metal ion might be taken as support for this hypothesis. However, if such an enediol derivative is formed as an intermediate, it does not accumulate in detectable quantities. It is established that CO_2 enters the carboxyl group of PGA, while the 1 position of pentose phosphate appears in the β position of the product; it would be of interest to establish, in experiments with C^{13} of the type introduced by Wood (32), that these labeled carbon atoms are present in the same PGA molecule. With the isolation of the carboxylation enzyme, the entire CO_2 fixation cycle in photosynthesis can be written in terms of known reactions. These have been reviewed elsewhere (5, 9, 33).

SUMMARY

In the presence of ribulose diphosphate and the carboxylation enzyme, CO_2 is fixed in the carboxyl group of phosphoglyceric acid. With ribulose diphosphate-1-C^{14} the isotope appears in the β-carbon atom of phosphoglyceric acid. 2 moles of PGA are formed for each mole of ribulose diphosphate added. No evidence for the reverse reaction has been obtained.

The carboxylation enzyme has been purified about 10-fold from spinach extracts. It behaves as a homogeneous protein (molecular weight about 300,000) on ultracentrifugation and electrophoresis. It required the addition of divalent metal ions and sulfhydryl compounds or chelating agents for full activity. No substrates other than ribulose diphosphate are utilized.

The product has been identified as phosphoglyceric acid on the basis of chromatographic behavior, degradation, and enzymatic analysis. No evidence for enediol or 3-ketopentose intermediates in the reaction has been obtained. Triose phosphate is excluded as an intermediate.

The rôle of the carboxylation enzyme in photosynthesis is discussed.

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FORMATION OF 3-PHOSPHOGLYCERIC ACID BY CARBON DIOXIDE FIXATION WITH SPINACH LEAF ENZYMES*

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The work of Calvin and his coworkers (1, 2) has established that 3-phosphoglyceric acid[1] is an early product of photosynthesis formed by a "dark" carboxylation reaction. Studies of Fager (3) with spinach chloroplasts have also demonstrated PGA formation as the result of a reaction of CO_2 with an unidentified acceptor. Horecker and Weissbach[2] were the first investigators to demonstrate the occurrence of a carboxylation reaction in soluble preparations of spinach leaf leading to the formation of carboxyl-labeled PGA, in the presence of $C^{14}O_2$ and ribose-5-phosphate, or to that of predominantly β-labeled PGA in the presence of CO_2 and ribose-5-phosphate-1-C^{14}.

Attempts in this laboratory to determine whether soluble spinach leaf preparations would bring about a reversal of the Horecker reaction, *i.e.* a decarboxylation or oxidative decarboxylation of PGA, in the presence of possible "glycolaldehyde" acceptors such as triose phosphate and hydrogen acceptors such as DPN+ or TPN+, gave negative results; there was no net production of CO_2, or exchange of $C^{14}O_2$ with PGA, under a variety of experimental conditions. In the course of these experiments it was noted that, in the presence of ribose-5-phosphate, the spinach preparations fixed

[1] The following abbreviations are used: PGA, 3-phosphoglyceric acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; DPN+, DPNH, TPN+, TPNH, oxidized and reduced di- and triphosphopyridine nucleotide respectively; Tris, tris(hydroxymethyl)aminomethane.

[2] Presented by B. L. Horecker at Atlantic City, April, 1954, before the American Society of Biological Chemists (4).

CO_2 to form 2 molecules of PGA per molecule of CO_2 utilized. With dialyzed enzyme preparations this reaction was dependent on the presence of ATP and Mg^{++}. Similar results were obtained independently by Quayle et al. (5), Weissbach et al. (6), and Racker and collaborators (cf. (7)). Further work of Horecker and his coworkers (8–10) led to the identification of ribulose-1,5-diphosphate as the CO_2 acceptor in PGA synthesis. This rôle of ribulose diphosphate had been postulated (2) and made likely (5) by Calvin and collaborators.

The present work has resulted in the separation from spinach leaf extracts of soluble enzyme preparations catalyzing the over-all Reactions 1 and 2.

(1)   Ribose-5-phosphate + ATP → ribulose-1,5-diphosphate + ADP

(2)   Ribulose-1,5-diphosphate + CO_2 + H_2O → 2 3-phosphoglyceric acid
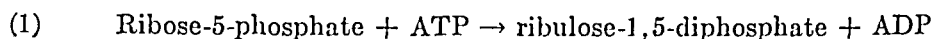
These systems will be referred to for convenience as the kinase and the carboxylation systems respectively. As shown by Horecker et al. (9, 10), Reaction 1 consists of (a) the reversible isomerization of ribose-5-phosphate to ribulose-5-phosphate, catalyzed by phosphoriboisomerase (11), and (b) the phosphorylation of ribulose-5-phosphate by ATP, to form ribulose-1,5-diphosphate and ADP, catalyzed by phosphoribulokinase.

The partial purification of enzymes concerned with Reactions 1 and 2 and the identification of the reaction products are reported in this paper.

Enzyme Assays

Kinase—Since 1 mole of acid is produced per mole of ribulose diphosphate formed, Reaction 1 was assayed manometrically by measuring the evolution of CO_2 in a bicarbonate buffer. The reaction mixture (in Warburg flasks) contained the following components (in micromoles) in a final volume of 1.0 ml.: KHCO₃, 40; L-cysteine, 10; reduced glutathione, 4; MgCl₂, 10; ATP, 30; ribose-5-phosphate, 30; and an amount of enzyme (in the side arm) which would bring about the evolution of from 10 to 100 μl. of CO_2 in 15 minutes. Controls without ATP and without ribose-5-phosphate were run simultaneously with the experimental samples to correct for ATPase and phosphatase activities. ATPase activity was mostly negligible, but there was some phosphatase activity on ribose-5-phosphate. The flasks were gassed with a mixture of 95 per cent N_2 and 5 per cent CO_2 and equilibrated at 30°. The pH was about 7.8. The reaction was started by tipping in the enzyme and followed for at least 15 minutes. Within the limits mentioned the evolution of CO_2 was proportional to enzyme concentration (Fig. 1, A). 1 unit of kinase activity is taken as the amount of enzyme catalyzing the evolution of 1.0 μl. of CO_2 per minute under the above conditions.

Carboxylation System—The fixation of $C^{14}O_2$ was the basis of the carboxylation system assay. The reaction mixture (in Warburg flasks) contained the following components (in micromoles) in a final volume of 1.5 ml.: in the main compartment, Tris buffer, pH 7.3, 100; KHCO_3, 20; L-cysteine, 20; MgCl_2, 5; ATP, 5; kinase, 5 units; and sufficient carboxylase to bring about the fixation of 1000 to 15,000 c.p.m. of $C^{14}O_2$ in 20 minutes; in the side arm, ribose-5-phosphate, 5; and $Na_2C^{14}O_3$ with 10^6 c.p.m. The flasks were gassed with 95 per cent N_2–5 per cent CO_2 and equilibrated at 30° as in the kinase assay. The reaction was started by tipping in the con-
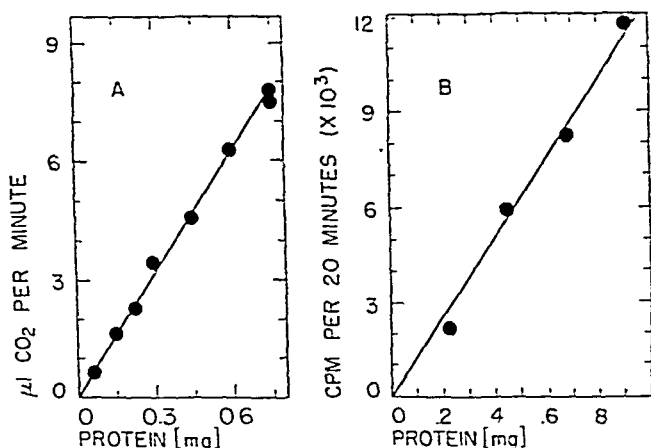

Fig. 1. Assay of kinase and carboxylation systems. *A*, rate of carbon dioxide evolution from bicarbonate buffer as a function of kinase concentration. The initial extract used had a specific activity of 11.8. *B*, rate of $C^{14}O_2$ fixation as a function of carboxylation enzyme concentration. The initial extract used had a specific activity of 13.

tents of the side arm and stopped, after incubation with shaking for 20 minutes at 30°, by the addition of 0.5 ml. of 1.0 N hydrochloric acid. 0.1 ml. aliquots of the mixture were used for the determination of fixed radioactivity. Within the limits mentioned the fixation of $C^{14}O_2$ was proportional to enzyme concentration (Fig. 1, *B*). 1 unit of carboxylation activity is taken as the amount of enzyme promoting the fixation of 1000 c.p.m. of $C^{14}O_2$ in 20 minutes under the above conditions.

Protein was determined spectrophotometrically by the method of Warburg and Christian (12).

Determination of 3-Phosphoglyceric Acid

PGA was determined enzymatically by either of two methods involving the use of phosphoglyceric kinase.

In one of the methods, based on the work of Axelrod and Bandurski (13), the formation of the hydroxamic acid of 1,3-diphosphoglyceric acid is measured on incubation of PGA with ATP and phosphoglyceric kinase in the presence of hydroxylamine. The reaction mixture contained the following components (in micromoles) in a final volume of 1.3 ml.: hydroxylamine, brought to pH 7.3, 200; MgCl₂, 5; ATP, 10; PGA, 0.2 to 1.5; and an excess of phosphoglyceric kinase. The samples were incubated for 90 minutes at 37°, and the reaction was stopped by the addition of 3.0 ml. of the ferric chloride-trichloroacetic acid reagent of Lipmann and Tuttle (14). The colorimetric determination was carried out within 30 minutes. A
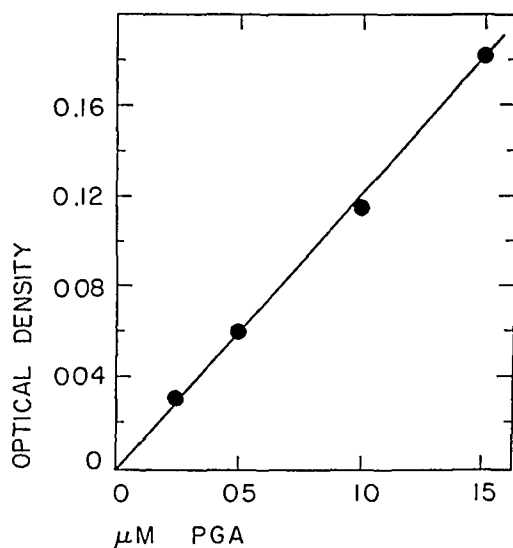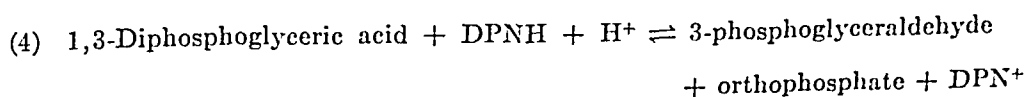

FIG. 2. Assay of phosphoglyceric acid by hydroxamic acid formation. Wavelength, 540 mμ. Details in the text.

standard curve with known amounts of PGA must be obtained with each assay, as the formation of hydroxamic acid is not quantitative. Within the limits and under the conditions mentioned the color obtained was a linear function of the PGA concentration (Fig. 2).

The other method is based on the spectrophotometric determination of the oxidation of DPNH in a coupled system containing PGA, ATP, phosphoglyceric kinase, DPNH, triosephosphate dehydrogenase, triosephosphate isomerase, and α-glycerol phosphate dehydrogenase (Reactions 3 to 6). The net result is Reaction 7. Under these conditions, 2 moles of DPN⁺ are formed per mole of PGA present.

(3)   3-Phosphoglyceric acid + ATP ⇌ 1,3-diphosphoglyceric acid + ADP

(4)   1,3-Diphosphoglyceric acid + DPNH + H⁺ ⇌ 3-phosphoglyceraldehyde

+ orthophosphate + DPN⁺
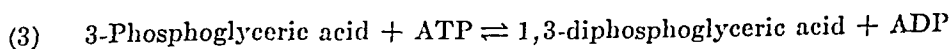
(5) 3-Phosphoglyceraldehyde $\rightleftharpoons$ dihydroxyacetone phosphate

(6) Dihydroxyacetone phosphate + DPNH + H$^+$ $\rightleftharpoons$ α-glycerol phosphate + DPN$^+$

(7) 3-Phosphoglyceric acid + ATP + 2DPNH + 2H$^+$ $\rightleftharpoons$ α-glycerol phosphate

+ orthophosphate + 2DPN$^+$

The assay was conducted in a cell of 1.0 cm. light path and 1.0 ml. volume in the Beckman spectrophotometer. The constituents (in micromoles) of the reaction mixture, in a final volume of 1.0 ml., consisted of the following: Tris buffer, pH 7.3, 100; MgCl$_2$, 3; L-cysteine, 5; ATP, 2.5; DPNH, 0.07; PGA, not exceeding 0.03; and excess of the enzymes detailed above.

Enzyme Fractionation

Large, turgid spinach leaves, trimmed of large veins, were washed and ground in a vegetable juice extractor. The juice was filtered through two layers of cheese-cloth, and 10 ml. of a neutralized solution of L-cysteine (15 mg. per ml.) were added per 100 ml. of juice. All subsequent operations were performed at 0°. The juice was adjusted to pH 7.3 with 1.0 N KOH and centrifuged for 40 minutes at 16,000 × g. The resultant supernatant fluid will be referred to as the extract.

To the extract was added solid ammonium sulfate until 0.2 saturation was reached. The precipitate was discarded and further ammonium sulfate added to 0.4 saturation. The precipitate was dissolved in one-quarter of the original extract volume of 0.05 M Tris buffer, pH 7.2, which was 0.01 M with respect to cysteine.

Kinase System—The carboxylation system is inactivated by acetone, while the kinase system may be obtained in good yield by fractional precipitation with this reagent. To the solution of the 0.2 to 0.4 ammonium sulfate precipitate was slowly added previously cooled (−30°) acetone until a concentration of 30 per cent (volume per volume) of acetone was reached. The addition of acetone was begun at −1°, the temperature being gradually lowered to −5° during the course of the addition. The precipitate was removed by centrifugation at −5°, and acetone was added to the supernatant fluid until a concentration of 50 per cent was reached. The precipitate obtained after centrifugation at −5° was suspended in one-fifteenth the volume of the original spinach extract of 0.05 M Tris buffer, pH 7.2, containing 0.01 M cysteine. This suspension was dialyzed against 20 volumes of the same buffer. Dialysis was continued for a total of 3 hours, after which the protein suspension was centrifuged and the residue discarded. The supernatant solution contains phosphoriboisomerase and phosphoribulokinase. An outline of the results of this fractionation is presented in Table I. It is of interest to note that crude extracts of

spinach petioles carefully freed of leaf tissue contain an abundance of kinase system with little carboxylation activity.

Carboxylation System—The solution of the 0.2 to 0.4 ammonium sulfate precipitate was brought to pH 6.3 with 1.0 N acetic acid. Aliquots of 20 ml. were quickly heated to 56° in stainless steel centrifuge cups and kept at this temperature for 3 minutes with stirring. The precipitated protein was removed by centrifugation and the pH of the supernatant solution adjusted to 6.7.

Chilled ($-30°$) 95 per cent ethanol was slowly added with stirring to the supernatant solution of the previous step until a concentration of 22 per cent (volume per volume) was reached. The precipitate was removed by centrifugation at $-5°$ and the ethanol concentration increased to 30 per cent. During the addition of ethanol the temperature was gradually lowered to $-4°$ at 22 per cent concentration and $-8°$ at 30 per cent concen-

TABLE I

Fractionation of Kinase System

Step	Volume	Protein	Units	Specific activity*	Yield
	ml.	*mg.*			*per cent*
Extract.....................................	150	8030	71,200	9	100
(NH₄)₂SO₄ ppt. (0.2–0.4).................	50	2930	39,000	13	55
Acetone ppt. (30–50%).......	10	130	18,000	138	25

* Units per mg. of protein.

tration. The precipitate obtained between 22 and 30 per cent ethanol was dissolved in one-tenth the original spinach extract volume of 0.02 M Tris buffer, pH 7.3.

The solution of the ethanol precipitate was brought to pH 6.5 with 1.0 N acetic acid, and 1.2 volumes of calcium phosphate gel (25 mg. of calcium phosphate per ml.) were added per 100 mg. of protein. After stirring for 10 minutes, the gel was collected by centrifugation and the supernatant solution was discarded. The gel was washed once with distilled water and eluted with an amount of 0.1 M Tris buffer, pH 7.3, equal to twice the volume of the added gel suspension. This eluate contains the carboxylation system with a specific activity 20 times higher than that of the extract. It also contains phosphoriboisomerase, but is free of phosphoribulokinase. The results of a typical fractionation are shown in Table II.

Reaction Products and Stoichiometry

Kinase—A mixture of 1.0 mmole of ATP, 1.0 mmole of ribose-5-phosphate, 1.0 mmole of MgCl₂, and 2.0 mmoles of L-cysteine was incubated

with a carboxylase-free kinase preparation for 30 minutes at 30°. The final volume was 100 ml.; the ATP, ribose-5-phosphate, and cysteine had previously been brought to about pH 8.0. At the end of the incubation the reaction mixture contained two chromatographically distinct components, each of which produced 2 moles of PGA per mole of $C^{14}O_2$ incorporated when assayed with the kinase-free carboxylase system. The reaction mixture was added without deproteinization to a Dowex 1-chloride column (0.8 × 8.0 cm.) and the resin eluted first with 0.1 M KCl. The major CO_2-fixing compound appeared in the fifth and sixth 100 ml. effluent fractions. This material contained organic phosphate, which was quantitatively liberated by phosphatase (15), and a ketose. After treatment with phosphatase the compound liberated 2 moles of phosphate per mole of ketose; the latter was determined by the cysteine-carbazole reaction (16), with

TABLE II

Fractionation of Carboxylation System

Step	Volume	Protein	Units	Specific activity*	Yield
	ml.	mg.			per cent
Extract....................................	270	8420	79,100	9	100
$(NH_4)_2SO_4$ ppt. (0.2–0.4)..................	64	2200	53,600	25	68
Heat step supernatant fluid........... ...	61	1170	47,800	41	60
Ethanol ppt. (22–30%)	25	184	24,900	135	32
$Ca_3(PO_4)_2$ gel eluate.....................	18	92	18,000	198	23

* Units per mg. of protein.

ribulose as standard, after enzymatic dephosphorylation. The absorption spectra of the colored compounds formed by the unknown ketose and authentic ribulose were identical. The isolated compound, therefore, appears to be the same as that described by Horecker, Hurwitz, and Weissbach (10) as ribulose-1,5-diphosphate.

The second component was eluted with 0.2 M KCl-0.015 N HCl and was found to contain approximately 20 per cent as much ketose as the first. This compound has not been further identified.

Carboxylation System—PGA was identified as the product of the CO_2 fixation reaction through isolation from a reaction mixture incubated with $C^{14}O_2$. A dialyzed ammonium sulfate fraction of spinach, containing both the kinase and carboxylation systems, was incubated with the following components (in micromoles) in a final volume of 20 ml.: Tris buffer, pH 7.3, 1200; ribose-5-phosphate, 150; $MgCl_2$, 100; ATP, 150; and $NaHCO_3$ (containing 3 × 10^7 c.p.m. of C^{14}), 30. The incubation time was 100 minutes and the temperature 30°. The reaction mixture was deproteinized

with 5 ml. of 10 per cent trichloroacetic acid. The fixed radioactivity in the protein-free supernatant solution amounted to 2.6 × 10⁷ c.p.m. 855 µmoles of PGA were added to this solution as inert carrier, and the crystalline acid barium salt of PGA was prepared and isolated as described by Neuberg and Lustig (17) with some modifications.[3] After four recrystallizations the specific radioactivity of the PGA remained constant

TABLE III

Analytical Data of Isolated C^{14}-PGA

Analyses performed on a sample of the crystalline acid barium salt dried to constant weight *in vacuo* over P_2O_5 at 50°.

Inorganic phosphate, %	0.16
Organic phosphate, %	8.88*
$[\alpha]_D^{20}$ of molybdate complex, *degrees*	−733†
Specific radioactivity of PGA, *c.p.m. per µmole*	2.6 × 10⁴
" " " " carboxyl, *c.p.m. per µmole*	2.6 × 10⁴

     * Calculated for $C_3H_5O_7PBa(2H_2O)$, 8.7 per cent.
     † Found by Meyerhof and Schulz (18), −745°.

TABLE IV

Stoichiometry of Carboxylation Reaction

Experiment No.	Substrate	$C^{14}O_2$ incorporated	PGA formed
		µmoles	*µmoles*
1	Ribose-5-phosphate	0.885	1.68*
			1.78†
2	Ribulose diphosphate‡	0.649	1.30†
3	" "	1.07	2.02†

     * Determined by the hydroxamic acid method.
     † Determined by the spectrophotometric method.
     ‡ This material is the compound described in the text as the major product of the kinase system. Kinase and ATP were not present in Experiments 2 and 3.

at 26,000 c.p.m. per µmole. The yield of crystalline acid barium salt (dried to constant weight *in vacuo* over $CaCl_2$) at this stage was 80 mg. For $C_3H_5O_7PBa(2H_2O)$ (mol. wt., 357.5) this corresponds to 224 µmoles of PGA. The material, which was essentially free of inorganic phosphate, contained 1 mole of bound phosphate per mole of PGA, and the molybdate complex (18) had the expected rotation. 4.1 per cent of the esterified phosphate was released on hydrolysis in 1.0 N HCl for 3 hours at 100°.
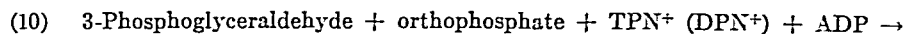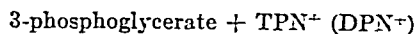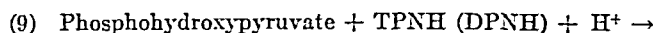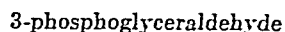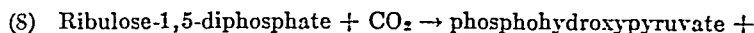
     [3] S. Ratner, unpublished. We are indebted to Dr. Ratner for the details of her method.

The analytical data are given in Table III. An aliquot of the labeled PGA was degraded with lead tetraacetate in glacial acetic acid in a Warburg manometric flask, and the CO_2 thus liberated was absorbed by potassium hydroxide in the center well. 1 mole of CO_2 was evolved per mole of PGA. The specific radioactivities of the resulting Na_2CO_3 and the starting PGA were equal on a molar basis (Table III). Thus, it appears that the product of the carboxylation reaction is D(−)3-phosphoglyceric acid and that the CO_2 incorporated is present exclusively in the carboxyl group.

The stoichiometry of the carboxylation reaction was investigated by comparing the amount of $C^{14}O_2$ fixed with the amount of PGA formed. The reaction was carried out as in the standard carboxylase assay, except that the mixture was incubated in an atmosphere of helium. In a control flask the reaction was stopped with acid at zero time to determine the specific radioactivity of the $C^{14}O_2$ initially present. The results, summarized in Table IV, show that 2 moles of PGA are formed per mole of CO_2 incorporated, in agreement with Reaction 2.
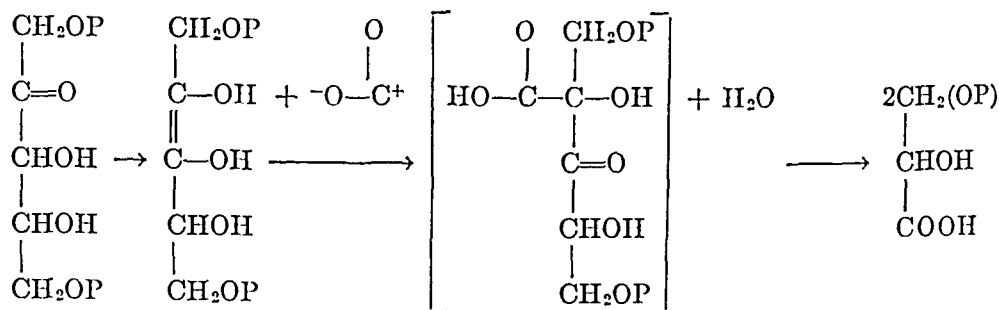
DISCUSSION

It appeared possible that Reaction 2 might be the result of Reactions 8 to 10. Reaction 8 bears some similarity to certain reactions catalyzed by transketolase (19, 20), while an enzyme, D-glyceric dehydrogenase, catalyz-

(8) Ribulose-1,5-diphosphate + CO_2 → phosphohydroxypyruvate +

3-phosphoglyceraldehyde

(9) Phosphohydroxypyruvate + TPNH (DPNH) + H^+ →

3-phosphoglycerate + TPN^+ (DPN^+)

(10) 3-Phosphoglyceraldehyde + orthophosphate + TPN^+ (DPN^+) + ADP →

3-phosphoglycerate + TPNH (DPNH) + H^+ + ATP

ing the reduction of hydroxypyruvate by DPNH to D-glycerate, a reaction analogous to Reaction 9, is widely distributed in higher plants and particularly active in green leaves (21). However, neither a DPN- (22) nor a TPN-specific (23) D-3-phosphoglyceraldehyde dehydrogenase, an enzyme required for the above sequence, was found in the purified carboxylation system preparations from spinach. These preparations did contain a TPN-specific 3-phosphoglyceraldehyde dehydrogenase which required no orthophosphate and was not stimulated by arsenate (24), but its activity was too low to account for the PGA-synthesizing activity of the spinach enzyme. As regards D-glyceric dehydrogenase, Fager (3) has pointed out that the enzyme was not present in those spinach fractions which he found most effective in fixing CO_2 into PGA.

An alternative mechanism proposed by Calvin (25), involving the ene-diol of ribulose-1,5-diphosphate, is presented. Whether the reaction ac-

$$
\begin{array}{ccc}
\begin{array}{l}
\text{CH}_2\text{OP} \\
| \\
\text{C}=\text{O} \\
| \\
\text{CHOH} \\
| \\
\text{CHOH} \\
| \\
\text{CH}_2\text{OP}
\end{array}
\rightarrow
\begin{array}{l}
\text{CH}_2\text{OP} \\
| \\
\text{C}-\text{OH} \\
\| \\
\text{C}-\text{OH} \\
| \\
\text{CHOH} \\
| \\
\text{CH}_2\text{OP}
\end{array}
+ {}^-\text{O}-\text{C}^+
\longrightarrow
\left[
\begin{array}{l}
\quad\;\;\;\text{O}\;\;\;\text{CH}_2\text{OP} \\
\quad\;\;\;| \quad | \\
\text{HO}-\text{C}-\text{C}-\text{OH} \\
\quad\;\;\;| \\
\quad\;\;\;\text{C}=\text{O} \\
\quad\;\;\;| \\
\quad\;\;\;\text{CHOH} \\
\quad\;\;\;| \\
\quad\;\;\;\text{CH}_2\text{OP}
\end{array}
\right]
+ \text{H}_2\text{O}
\longrightarrow
\begin{array}{l}
2\text{CH}_2(\text{OP}) \\
| \\
\text{CHOH} \\
| \\
\text{COOH}
\end{array}
\end{array}
$$

tually proceeds in separate steps catalyzed by distinct enzymes cannot be decided from our data.

As indicated by its apparent irreversibility, the carboxylation reaction must proceed with a large negative free energy change which must be largely contributed by the cleavage of the bond between carbons 2 and 3 of ribulose diphosphate. The biological significance of this unique feature in CO_2 fixation reactions is obvious, as it insures the rapid and efficient utilization of CO_2 by autotrophic organisms at the low prevailing tensions of this gas. The carboxylation system described here is not the exclusive property of green cells, since the occurrence of Reactions 1 and 2 in extracts of a strain of *Thiobacillus*, a non-photosynthesizing autotroph, has been established in joint experiments with M. Santer and W. Vishniac.

The carboxylation leading to PGA is a "dark" reaction, and the only effect of light in the case of green cells (or of the oxidation of inorganic compounds in other autotrophic organisms) is to provide hydrogens for the reduction of PGA to the carbohydrate level, *i.e.* to triose phosphate. Since this reaction involves phosphoglyceric kinase (Reaction 3) and D-3-phos-phoglyceraldehyde dehydrogenase (Reaction 4), the demonstration of the photochemical reduction of pyridine nucleotides by chloroplast prepara-tions (26) and of the phosphorylation of ADP (also essential for the phos-phoribulokinase reaction) through the oxidation of photochemically re-duced pyridine nucleotides (27) or other intermediates (28) provides the required link with the photochemical reaction.

It is of interest that, as postulated by Gaffron and Fager (29), the car-boxylation leading to PGA is the only carboxylation necessary for a com-plete photosynthesis (or chemosynthesis) cycle. In the light of present knowledge the CO_2 acceptor, ribulose diphosphate, can be regenerated from triose and hexose phosphates through reactions involving, besides aldolase and phosphotriose isomerase, fructose diphosphatase, transketo-lase, transaldolase, phosphoriboisomerase, and phosphoribulokinase (7, 9, 10, 19, 20, 30–32), all of which appear to be widely distributed in nature.

Materials and Methods

The crystalline acid barium salt of D-3-phosphoglyceric acid, kindly supplied by Dr. S. Ratner, was used as a reference standard. Ribulose was prepared by the method of Glatthaar and Reichstein (33). All other compounds were obtained commercially.

Phosphoglyceric kinase from yeast was prepared as described by Bücher (34). The preparation, which was brought to the stage prior to the addition of alumina gel, was contaminated with sufficient α-glycerol phosphate dehydrogenase and phosphotriose isomerase to be utilized for the spectrophotometric assay of PGA described in a previous section. For use with the hydroxamic acid assay, phosphoglyceric kinase was prepared from pea seeds by the method of Axelrod and Bandurski (13). Crystalline D-3-phosphoglyceraldehyde dehydrogenase (35) was prepared from rabbit muscle by Dr. M. Schwartz. Assays for 3-phosphoglyceraldehyde dehydrogenase were carried out spectrophotometrically in the presence of arsenate with fructose-1,6-diphosphate and aldolase as a source of D-3-phosphoglyceraldehyde. Crystalline aldolase (36) was a gift of Dr. E. Racker.

Radioactivity was determined by plating samples on aluminum planchets containing disks of lens paper embedded in agar. The samples were dried under an infra-red lamp and counted with a windowless gas flow counter. Organic phosphate was converted to orthophosphate by ashing as described by LePage (37) and orthophosphate determined by the method of Lohmann and Jendrassik (38).

SUMMARY

Two soluble enzyme preparations have been obtained from spinach leaves catalyzing (a) the formation of a ketopentose phosphate, characterized as ribulose diphosphate, from ribose-5-phosphate and adenosine triphosphate, in the presence of magnesium ions, and (b) the reaction of ribulose diphosphate with $C^{14}O_2$ to form D-3-phosphoglyceric acid-1-C^{14}. 2 molecules of phosphoglyceric acid are formed per molecule of CO_2 utilized. No indications could be obtained for reversibility of this reaction. The enzymes concerned with reactions (a) and (b) have been separated from each other and partially purified. However, the work of Horecker and coworkers shows that reaction (a) consists of the isomerization of ribose-5-phosphate to ribulose-5-phosphate and the phosphorylation of the latter by adenosine triphosphate catalyzed by two distinct enzymes. The mechanism and significance of the carboxylation reaction are discussed.

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ENZYMATIC REACTIONS OF FLUOROACETATE AND FLUOROACETYL COENZYME A*

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The toxic effect of fluoroacetate generally has been considered to be due to the formation of fluorocitrate *in vivo* (2–4), resulting in aconitase inhibition. Recently, Peters *et al.* (5) isolated a fluorotricarboxylic acid from kidney homogenates incubated with fumarate and fluoroacetate. Peters and Wakelin established its identity as fluorocitrate (6), by comparison with the synthetic compound. However, fluorocitrate synthesis in a partially purified enzyme system could not be demonstrated (7).

In the experiments to be described, we have investigated (*a*) the activation of fluoroacetate and (*b*) the enzymatic activity of FAc-CoA.[1] The results indicate that the metabolic pathway of fluoroacetate is similar to that of acetate.

Materials and Methods

Fluoroacetyl chloride was prepared by treating sodium fluoroacetate with PCl$_5$ (8) and redistilling the product through a packed column. The fraction that distilled at 69.5–70.0° was treated further (9) with sodium fluoroacetate (dried *in vacuo* over H$_2$SO$_4$) to yield fluoroacetic anhydride (b.p. 88.5–90.0° per 11 mm.). A portion of the anhydride was converted to fluoroacethydroxamic acid and compared in ascending chromatography with acethydroxamic acid. In each of several solvent systems studied, fluoroacethydroxamic acid migrated more slowly. The best separation was attained in phenol-H$_2$O (3:1) in which fluoroacethydroxamic acid migrated with an R_F of 0.76 while the R_F for acethydroxamic acid was 0.86.

[1] The following abbreviations are used: Ac-CoA, *S*-acetyl coenzyme A; FAc-CoA, *S*-fluoroacetyl coenzyme A; —SH, free sulfhydryl; Ac, sodium acetate; FAc, sodium fluoroacetate; ATP, adenosine triphosphate; CoA, coenzyme A; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; P, product of reaction.

Ac-CoA and FAc-CoA were prepared from their respective anhydrides by a modification (10) of the method of Simon and Shemin (11). The products were extracted with ether several times and immediately lyophilized to give a white powder. Both CoA derivatives were determined by hydroxamic acid formation (12, 13) and liberation of —SH on alkaline hydrolysis (14). Standard acethydroxamic acid was prepared from acetic anhydride and checked against a similar preparation from acetamide. Cysteine hydrochloride was used as a standard in the —SH determinations. The FAc-CoA preparation contained considerable free —SH which increased slowly upon standing at 3°. Similar instability has been reported for FAc-CoA prepared by another method (15). The absorption curve for FAc-CoA appeared to be identical with that of Ac-CoA as given by Stadtman (16).

In all the enzyme studies, 0.02 M sodium or potassium bicarbonate extracts of pigeon liver acetone powders were used (17). The extracts were fractionated with $(NH_4)_2SO_4$ (18), acetone, and protamine (19) as indicated in the respective experiments. The fractions were dialyzed against 1000 volumes of 0.02 M $NaHCO_3$-0.002 M cysteine-0.05 per cent KCl adjusted to pH 8.0 by addition of Na_2CO_3.

Sulfanilamide was determined according to Bratton and Marshall (20), and p-nitroaniline acetylation was followed spectrophotometrically by decrease in absorption at 420 mμ (21). Protein was estimated by a modification of the Folin-Ciocalteu method (22).

Citrate and "fluorocitrate" were determined by a modification of the method of Saffran and Denstedt. After incubation, the residual oxalacetate was destroyed by heating the mixture in a water bath at 90° for 5 minutes. Trichloroacetic acid was added to bring the final concentration to 5 per cent and the precipitated protein was centrifuged. Suitable aliquots were analyzed as described (23), each determination being controlled by appropriate blanks and citrate recoveries in the expected range.

ATP (95 per cent, Nutritional Biochemicals Corporation) and CoA were obtained commercially. In the synthesis of acetyl and fluoroacetyl coenzyme A a preparation (Pabst) containing 1 μmole of CoA per mg. (24) was used. In all other experiments, a porcine liver CoA concentrate (Armour) assayed at 13 Lipmann units per mg. was used. We are indebted to the Monsanto Chemical Company for gifts of technical sodium fluoroacetate (Compound 1080).

Results

Activation of Fluoroacetate

When fluoroacetate was added in the sulfanilamide-acetylating system, the increase in amine acetylation indicated that fluoroacetate was

activated, presumably with the formation of FAc-CoA (Table I). The amount acetylated increased with increasing fluoroacetate concentration up to a limiting value. In contrast to acetate, high concentrations of fluoroacetate are required to yield significant acylation. Similar results were obtained for the formation of hydroxamic acid (Table II) with either crude (Monsanto) or purified fluoroacetate (25).

In experiments in which the substrate concentration was kept below that giving maximal acetylation, glutathione was more effective than cysteine in promoting acetylation (Table III). Since glutathione has been shown to serve as a stable acetyl acceptor (26), its enhancement of the

TABLE I

Acetylation of Sulfanilamide

Experiment No. and additions		Amount acetylated	Excess over blank
	$\mu moles$	γ	γ
I. None		12.5	
" Ac	1	39.3	26.8
" "	4	44.5	32.0
" "	20	47.5	35.0
" "	30	47.8	35.3
II. None		33.6	
" FAc	20	37.9	4.3
" "	40	44.5	10.9
" "	60	49.8	16.2
" "	80	49.5	15.9

All tubes contained 69.0 γ (0.4 μmole) of sulfanilamide, 20 μmoles of citrate, 4 μmoles of ATP, 20 μmoles of cysteine, 7.7 units of CoA, 0.3 cc. of pigeon liver extract, and 100 μmoles of Tris buffer (pH 8.1). Incubated for 90 minutes at 30°.

reaction may involve storage of the "activated" acetate to be subsequently released to the amine-acetylating system.

Activation of fluoroacetate in "citrate" synthesis was demonstrated in a 35 to 70 per cent $(NH_4)_2SO_4$ fraction (18) of pigeon liver (Table IV). As in the amine acetylation, this reaction with fluoroacetate seemed to parallel that of acetate, with the exception that high concentrations of CoA and ATP were required for significant activity.

Fluoroacetyl Coenzyme A

Since the experimental evidence indicated that fluoroacetate underwent both carboxyl and methyl activation, it appeared desirable to test the action of FAc-CoA with fractionated enzyme preparations. Fig. 1 shows a comparison of Ac-CoA and FAc-CoA in the acetylation of p-nitroaniline with a 48 to 78 per cent acetone fraction of pigeon liver. An optical

density decrease of 0.150 is equivalent to 0.025 μmole acetylated. Addition of Ac-CoA at zero time (Curve B) gave 58 per cent acetylation (relative to Ac-CoA concentration). Further addition of enzyme increased the acetylation to 71 per cent, indicating that the substrate was not a

TABLE II

Hydroxamic Acid Formation

Experiment No. and additions		Hydroxamic acid	Excess over blank
	μmoles	*μmoles*	*μmoles*
I. None		1.92	
" Ac	50	4.95	3.03
" "	150	5.04	3.12
" FAc	50	2.40	0.48
" "	100	2.62	0.70
" "	150	2.70	0.78
II. None		1.01	
" Ac	50	3.39	2.38
" FAc	50	1.35	0.34
" "Purified" FAc	40	1.22	0.21

All tubes contained 750 μmoles of hydroxylamine (neutralized with KOH to pH 7.0) and 320 μmoles of Tris buffer (pH 8.1). In Experiment I the additions were 50 μmoles of MgCl$_2$, 50 units of CoA, 30 μmoles of ATP (15 μmoles at zero time and 15 μmoles at 30 minutes), 40 μmoles of glutathione (20 μmoles at zero time and 20 μmoles at 30 minutes), 0.35 cc. of pigeon liver extract, and water to a final volume of 1.85 cc. Incubated 150 minutes at 25°. In Experiment II, the additions were 25 μmoles of MgCl$_2$, 20 units of CoA, 16 μmoles of ATP (8 μmoles at zero time and 8 μmoles at 30 minutes), 30 μmoles of glutathione (15 μmoles at zero time and 15 μmoles at 30 minutes), 0.20 cc. of pigeon liver extract, and water to a final volume of 1.6 cc. Incubated for 120 minutes at 28°.

TABLE III

Effect of Glutathione on Acetylation

Experiment No. and additions (30 μmoles each)	Amount acetylated		Δ
	With 20 μmoles cysteine	With 20 μmoles glutathione	
	γ	*γ*	*γ*
I. None	31.8	35.4	+3.6
" Ac	42.2	43.2	+1.0
" FAc	34.9	39.6	+4.7
II. None	40.4	57.5	+17.1
" Ac	67.4	68.4	+1.0
" FAc	50.8	62.2	+11.4

Conditions as in Table I except for Experiment II in which 50 per cent higher quantities of sulfanilamide and citrate were used.

TABLE IV

Fluoroacetate in Citrate Synthesis

Experiment No. and additions (50 μmoles each)	"Citrate" formed	Excess over blank
	μmoles	*μmoles*
I. None	0.49	
" Ac	0.94	0.45
" FAc	0.52	0.03
II. None	0.31	
" Ac	1.82	1.51
" FAc	0.49	0.18

All tubes contained 10 μmoles of glutathione, 10 μmoles of $MgCl_2$, 10 units of CoA, 25 μmoles of oxalacetic acid. In Experiment I other additions were 5 μmoles of ATP, 35 to 70 per cent $(NH_4)_2SO_4$ fraction (3.7 mg. of protein), and water to a final volume of 1.1 cc. All components were neutralized to pH 7.0 with KOH. In Experiment II other additions were 15 μmoles of ATP, 240 μmoles of Tris buffer (pH 8.0), $(NH_4)_2SO_4$ fraction (5.5 mg. of protein), and water to a final volume of 1.3 cc. Incubated for 90 minutes at 25°.
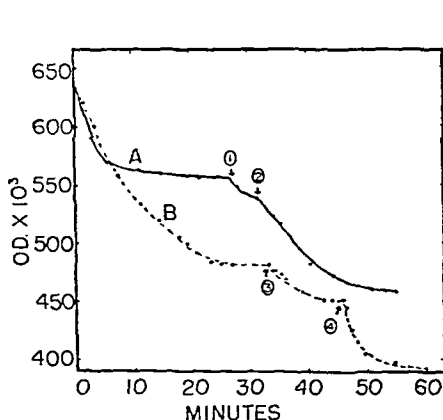

FIG. 1   FIG. 2

FIG. 1. Acetylation of *p*-nitroaniline. The samples contained 200 μmoles of phosphate buffer (pH 6.8), 5 μmoles of sodium thioglycolate, 5 μmoles of ethylenediaminetetraacetic acid (titrated with NaOH to pH 8.2), 0.10 μmole of *p*-nitroaniline, 0.05 cc. of enzyme (A78) equivalent to 10 mg. of liver powder, and water to a volume of 1.0 cc. Temperature 25°. Curve A, reaction started by adding 0.038 μmole of FAc-CoA. At Arrow 1, addition of 0.05 cc. of enzyme; at Arrow 2, addition of 0.0215 μmole of Ac-CoA. Curve B, reaction started by adding 0.043 μmole of Ac-CoA. At Arrow 3, addition of 0.05 cc. of enzyme; at Arrow 4, addition of 0.019 μmole of FAc-CoA. All optical density readings are corrected for dilution after additions.
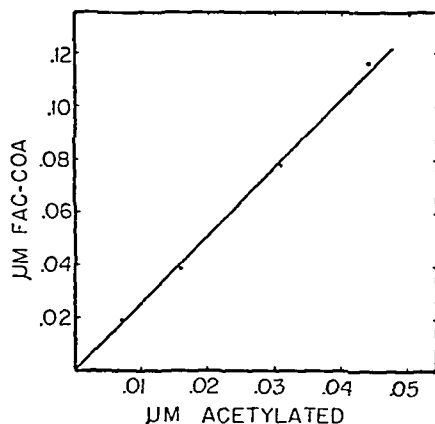
FIG. 2. Acetylation of sulfanilamide with varying concentrations of FAc-CoA. All conditions are as in Fig. 1, except for the replacement of *p*-nitroaniline by 0.1 μmole of sulfanilamide. Incubated for 60 minutes at 25°.

limiting factor. When the reaction reached apparent equilibrium, FAc-CoA was added, whereupon acylation continued, demonstrating that the enzyme concentration was not limiting. The reactions were then carried out in reverse order (Curve A). In this case FAc-CoA acylated 34 per cent and increased to 41 per cent on addition of enzyme. Subsequent addition of Ac-CoA continued the reaction. The amount acetylated was found to be directly proportional to the concentration of FAc-CoA (Fig. 2), provided the enzyme concentration was constant.

Activity in "citrate" synthesis was tested with Fraction A-40 (30 to 40 per cent acetone) of pigeon liver. Acetoacetate-synthesizing enzyme was removed by precipitation with protamine (19). As in the amine acetylation reaction, FAc-CoA was far more active in "citrate" synthesis (Table V) than was suggested from the activation experiments.

TABLE V

Fluoroacetyl Coenzyme A in Citrate Synthesis

Experiment No. and additions		"Citrate" formed
	$\mu mole$	$\mu mole$
I. Ac-CoA	0.27	0.19
" None		0.02
II. FAc-CoA	0.38	0.16
" None		0.00

All tubes contained 25 μmoles of phosphate buffer (pH 7.3), 8 μmoles of oxalacetic acid (neutralized with KOH to pH 7.4), citrate-synthesizing enzyme (0.9 mg. of protein), and water to a total volume of 0.5 cc. Incubated for 30 minutes at 25°.

DISCUSSION

FAc-CoA appears to be an active intermediate in fluorocitrate synthesis. The slightly decreased activity (relative to Ac-CoA) in amine acetylation is due, at least in part, to the presence of large amounts of free CoA in the preparations. Brady has independently demonstrated similar reactivity of FAc-CoA, extending his studies to several additional CoA-mediated transfers (27). The degree of activation of free fluoroacetate is less than might be expected from the reactivity of FAc-CoA.

Neither Ac-CoA nor FAc-CoA afforded quantitative acylation of *p*-nitroaniline. Since the reaction is resumed with addition of either enzyme or substrate, it is clear that neither enzyme nor substrate is destroyed, nor is the effect due to a reaction equilibrium. The remaining possibility is product inhibition. This seemed quite likely, since Tabor *et al.* (21) found that CoA decreases the rate of the acetylation reaction. Further reaction with addition of substrate requires that the inhibition be reversible, *i.e.*

(1) $E + S \rightleftharpoons ES \rightarrow E + P$

(2) $P + E \rightleftharpoons EP$

The initial reaction proceeds until the product to substrate ratio is such that not enough free enzyme is available to give a significant reaction; *i.e.*, the equilibrium of Reaction 2 is far to the right. At this point, if the enzyme concentration is increased, the reaction continues until a new product to substrate ratio has been reached. Further addition of substrate renews the reaction until the same product to substrate ratio is restored. As shown in Fig. 1, when FAc-CoA was added to the tube previously containing Ac-CoA, the acetylation proceeded to 51 per cent (based on FAc-CoA concentration). Conversely, when Ac-CoA was added to the tube previously containing FAc-CoA, acetylation proceeded to 61 per cent (relative to Ac-CoA concentration). The cross-addition

TABLE VI

Additive Reactions of Ac-CoA and FAc-CoA in Amine Acetylation

Order	Experiment A		Experiment B	
	Addition	Per cent acetylated	Addition	Per cent acetylated
	µmole		*µmole*	
Primary	Ac-CoA, 0.043	71	FAc-CoA, 0.038	41
Secondary	FAc-CoA, 0.019	51	Ac-CoA, 0.0215	61

Data calculated from Fig. 1.

experiments demonstrate that there was no inhibition of the Ac-CoA reaction by FAc-CoA and that both substrates react additively. A compilation of the data taken from Fig. 1 is presented in Table VI.

By the theoretical considerations noted above, secondary addition of either Ac-CoA or FAc-CoA should give a similar "per cent acetylated" as in primary addition. That this is true can be seen from the fact that the total "per cent acetylated" on primary addition exactly equals that of secondary addition. The greater acetylation with FAc-CoA upon secondary addition (Experiment A) relative to primary addition (Experiment B) is apparently an effect of the total substrate, *i.e.* residual Ac-CoA and added FAc-CoA. A similar explanation would apply for secondary addition of Ac-CoA. Further evidence for competitive product inhibition is found in Fig. 2 where the amount of acylation is directly proportional to the initial concentration of FAc-CoA, giving the same product to substrate ratio for a given concentration of enzyme. Product inhibition has also been reported for the analogous glycine *N*-acylase reaction (28). However, the data in the glycine reaction indicated non-competitive inhibition.

SUMMARY

1. Fluoroacetate has been shown to be enzymatically activated and to function in both amine acetylation and "citrate" synthesis. Glutathione is more effective than cysteine in promoting the acetylation.

2. Fluoroacetyl coenzyme A has been synthesized and shown to have activity comparable to acetyl coenzyme A in both amine acetylation and "citrate" synthesis.

3. Acetyl coenzyme A and fluoroacetyl coenzyme A do not react quantitatively in the acetylation of p-nitroaniline. Evidence is presented to indicate that the incomplete reaction is the result of competitive product inhibition.

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ENZYMES AND COENZYMES OF THE PYRUVATE
OXIDASE OF PROTEUS*

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The oxidative decarboxylation of pyruvate has been shown to occur by
several distinct pathways. The first cell-free system studied was that
from *Lactobacillus delbrueckii*, which produces acetyl phosphate (1) without
the intermediate formation of acetyl coenzyme A (2). The dismutase
systems from *Streptococcus faecalis* and from *Escherichia coli* produce acetyl
coenzyme A (3), but the pyruvate oxidase system from *Proteus vulgaris*
produces free acetate without the intermediate formation of acetyl phos-
phate (4) or acetyl coenzyme A (5).

The pyruvate oxidase of *P. vulgaris* consists of two separable protein
components (6) which have been identified as a particulate autoxidizable
cytochrome system (Component 1) and a soluble enzyme (Component 2),
which catalyzes the following reaction in the presence of diphosphothi-
amine (DPT) and an appropriate electron acceptor: pyruvate $+ H_2O \rightarrow$
acetate $+ CO_2 + 2H^+ + 2e$. Either 2,6-dichlorophenolindophenol or
ferricyanide may serve directly as an electron acceptor. Oxygen is con-
sumed only when Component 1 is present to serve as an intermediate elec-
tron carrier. No other coenzyme in addition to DPT has been demon-
strated to function in this system. A brief report of some of these results
has appeared (7).

Test Systems

A. Oxygen Consumption—Conventional manometric techniques were
used at a temperature of 37°. Each flask contained 0.02 M potassium phos-
phate buffer, pH 6.0, 100 γ of DPT, 200 γ of $MnSO_4 \cdot H_2O$, 100 μmoles of
potassium pyruvate, enzyme fractions, and water to 3.0 ml. The center
well contained 0.2 ml. of 20 per cent KOH. The rate of oxygen consump-
tion was calculated from the second 10 minute period after the addition of
pyruvate.

B. Reduction of Ferricyanide—The reaction mixture contained 0.03 M

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potassium phosphate buffer, pH 6.0, 50 γ of DPT, 100 γ of $MnSO_4 \cdot H_2O$, 50 μmoles of potassium pyruvate, 25 μmoles of potassium ferricyanide, enzyme fractions, and water to 1.0 ml.; temperature, 25°. The rate of ferricyanide reduction was calculated from the amount of ferrocyanide produced in the first 10 minutes after the addition of ferricyanide.

C. Reduction of 2,6-Dichlorophenolindophenol (2,6-DCP)—The reaction mixture was the same as that of Test System A, except that 200 γ of 2,6-DCP were added; temperature, 25°. The rate of 2,6-DCP reduction was calculated from the second 1 minute interval after the addition of 2,6-DCP by observing the decrease in optical density at 600 mμ in a 1 cm. cuvette.

1 unit of Component 2 is equivalent to the utilization of 0.5 μl. of oxygen per hour in the presence of an excess of Component 1 with Test System A. 1 unit of Component 1 is that amount of Component 1 which permits half maximal velocity of 1 unit of Component 2 in Test System A.

Enzyme Fractionation and Purification

Cells were grown, collected, and disrupted by sonic oscillation as previously described (5). Unless otherwise indicated, all operations were carried out at 4°; enzyme solutions or suspensions were brought to the indicated decimal fraction of saturation with ammonium sulfate by the addition of a saturated solution of ammonium sulfate; centrifugation was at 25,000 × g in the SS-1 head of the Servall refrigerated centrifuge.

Purification of Component 2. *Step 1*—Cell fragments and intact cells were removed from the suspension by centrifuging for 30 minutes. Fraction 1, yield (from 65 gm. of wet cell paste): 3920 mg. of protein, 693,000 units, per cent recovery 100, specific activity 176.

Step 2—Fraction 1 was brought to 0.62 of saturation with ammonium sulfate, held for 2 hours, and centrifuged for 25 minutes. The supernatant fluid was discarded and the precipitate was suspended in a minimal amount of distilled water and dialyzed against 10 liters of distilled water for 20 hours. Fraction 2, yield: 3180 mg. of protein, 565,000 units, per cent recovery 82, specific activity 178.

Step 3—Fraction 2 was brought to 0.38 of saturation with ammonium sulfate, held for 1 hour, and then centrifuged for 20 minutes. The precipitate was washed once in 0.38 saturated ammonium sulfate, the washed precipitate containing Component 1; its treatment is described below. The supernatant fluid and wash liquid were combined, and solid ammonium sulfate was added to saturation. After 1 hour the protein suspension was decanted from the excess ammonium sulfate and centrifuged for 20 minutes. The supernatant fluid was discarded. The precipitate was dissolved in distilled water and dialyzed against 2 liters of distilled water for 16 hours. After dialysis, one-ninth volume of 0.20 M potassium phosphate

buffer, pH 6.0, was added to the enzyme solution, and it was heated at 58–60° for 10 minutes. The denatured protein was removed by centrifuging for 30 minutes. The supernatant fluid contained the active material. Fraction 3, yield: 421 mg. of protein, 440,000 units, per cent recovery 64, specific activity 1040.

Step 4—Fraction 3 was brought to 0.51 of saturation with ammonium sulfate, held for 1 hour, and centrifuged for 20 minutes. The precipitate was discarded, and the supernatant fluid was brought to 0.70 saturation by adding solid ammonium sulfate. After 1 hour the suspension was centrifuged for 20 minutes. The supernatant fluid was discarded; the precipitate was dissolved in a small volume of distilled water and dialyzed against 2 liters of distilled water for 16 hours. The precipitate which was

TABLE I

Summary of Purification of Component 2

Fraction No.		Protein	Units	Recovery	Specific activity
		mg.		*per cent*	
1	Cell-free extract	3920	693,000	100	176
2	0.62 saturated ammonium sulfate ppt.	3180	565,000	82	178
3	0.38 " " " supernatant fluid, heated	421	440,000	64	1,040
4	Dialyzed and washed 0.51–0.70 saturated ammonium sulfate ppt.	40	238,000	34	5,950
5	Ppt. from dialyzed salt solution of Fraction 4	7.4	186,000	27	25,170

Component 2 activity determined in Test System A in the presence of excess Component 1 (5800 units, 5 mg. of protein).

formed in the dialysate contained 80 per cent of the remaining activity. It was collected by centrifuging for 20 minutes and washed twice, each time with 10 ml. of distilled water. Fraction 4, yield: 40 mg. of protein, 238,000 units, per cent recovery 34, specific activity 5950.

Step 5—Fraction 4 was suspended in 11 ml. of 1 per cent KCl. The suspension was shaken every 20 minutes for 4 hours, then centrifuged for 20 minutes, and the precipitate discarded. Phosphate buffer at pH 6.5 was added to the supernatant fluid to a final concentration of 0.033 M. Fraction 5, yield: 7.4 mg. of protein, 186,000 units, per cent recovery 27, specific activity 25,170. The purification of Component 2 is summarized in Table I.

Treatment of Component 1—The precipitate obtained at 0.38 saturation with ammonium sulfate (see Step 3 above) was resuspended in distilled water and dialyzed against 10 liters of distilled water. Component 1 at this stage is always contaminated with small amounts of Component 2,

which can be removed by adsorption on calcium phosphate gel. Component 1 can also be freed of Component 2 by precipitation of Component 1, which occurs after alternate freezing and thawing. The latter treatment is preferred since more than 80 per cent of Component 1 activity is recoverable. The dialyzed suspension of the 0.38 saturated ammonium sulfate precipitate was kept in a deep freeze at $-17°$ for 5 days and thawed once every 24 hours. Component 1 was then precipitated by centrifuging for an hour. It was suspended in distilled water and evenly dispersed by sonic oscillation at 9 kc. per second for 8 minutes.
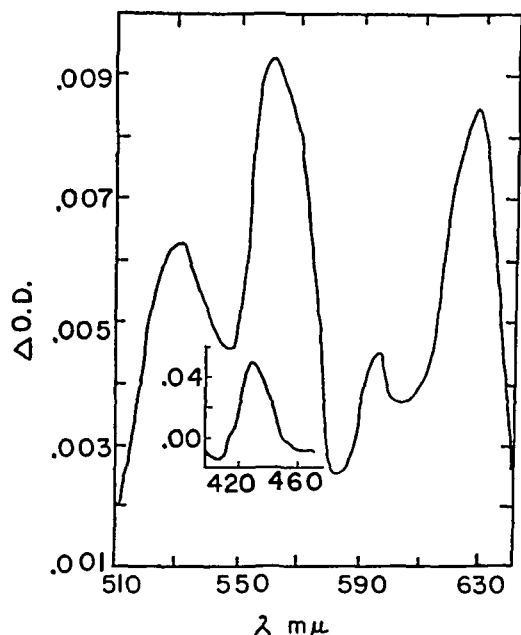

FIG. 1. Spectrum of the difference in absorption between oxidized and reduced Component 1. Test System A. The inset shows the Soret bands.

Functions and Properties of Protein Components

Component 1 is reddish brown and shows a marked Tyndall effect. The active material is associated with particles sedimentable in a centrifugal field of $60,000 \times g$. Component 1 is comparatively resistant to physical agents (5). The cytochrome system of Component 1 is autoxidizable when reduced by pyruvate in the presence of Component 2 in Test System A. Fig. 1 shows the spectrum of the difference in absorption of Component 1 between its oxidized state and enzymatically reduced state, measured in the recording spectrophotometer of Chance (8) by Dr. Lucile Smith. The same spectrum is obtained when hydrosulfite is the reducing agent. Distinct peaks occur at 627 and 560 mμ. There is a lower peak at 595 mμ. The fused β-bands have a single peak at 530 mμ. The Soret band has a peak at 429 mμ with a shoulder at 442 mμ. The peaks due to the difference

in absorption between the oxidized and reduced states appear only when the oxygen tension in one cuvette is zero. These peaks indicate the presence and functioning in Component 1 of cytochromes b_1, a_2, and possibly a_1.

The most highly purified Component 2 preparations are water-clear. This component is soluble in dilute salt solution but insoluble in distilled water, suggesting that it is a globulin. In the purified form it loses 50 per cent of its activity in 48 hours at 4° and is completely inactivated by freezing. However, in 0.033 M phosphate buffer, pH 6.5, it may be stored at 4° for a week without losing more than 10 per cent of its activity.

Component 2 reduces 2,6-DCP and ferricyanide in Test Systems C and B respectively. It does not permit oxygen uptake in Test System A unless

TABLE II

Inhibition of Action of Component 2

Reaction mixture	2,6-Dichlorophenolindophenol reduced per hr., μmoles per mg. protein
Complete.	11.6
" — Component 2	0.0
" — pyruvate	0.0
" — diphosphothiamine	0.0
" + 0.00001 M HgCl₂	0.0
" + 0.03 M NaAsO₂	11.1

The complete system consisted of Test System C with 55 γ of 2,6-dichlorophenolindophenol and 29 γ of Component 2 protein.

Component 2 is added (Table III). The DPT concentration required for half maximal rate in the reduction of 2,6-DCP by Component 2 is the same as that required for the aerobic oxidation of pyruvate by the *P. vulgaris* pyruvate oxidase preparation of Stumpf (4). Component 2 activity is strongly inhibited by mercuric chloride and unaffected by sodium arsenite (Table II). A product recovery showed that 30 μmoles of 2,6-DCP were reduced by Component 2 with the formation of 27.8 μmoles of acetate and 24 μmoles of carbon dioxide.

Component 2, in the presence of an excess of Component 1, oxidizes pyruvate at similar rates with either oxygen, ferricyanide, or 2,6-DCP as the final oxidant; however, the concentration of Component 1 necessary for half maximal rate of pyruvate oxidation varies with the electron acceptor (Table III). This indicates that the rate at which Component 2 oxidizes pyruvate and reduces the cytochromes of Component 1 is independent of the manner of reoxidation of Component 1, although the rate

of reoxidation of Component 1 is affected by the choice of electron acceptors. Ferricyanide is the most efficient oxidant.

The presently available data suggest that there are few, if any, similarities between the acetate-generating pyruvate oxidase of *P. vulgaris* and acetyl phosphate- or acetyl coenzyme A-generating pyruvate oxidases of other bacteria. The acetyl phosphate-generating system of *L. delbrueckii* apparently consists of a single protein requiring flavin adenine dinucleotide (FAD) and DPT (9). Coenzyme A (CoA) does not function (2). The acetyl CoA-generating oxidases are multienzyme systems requiring, in addition to CoA, diphosphopyridine nucleotide (DPN), DPT, and lipoic acid

TABLE III

Comparison of Electron Acceptors

System	Pyruvate oxidized per hr. in presence of		
	O_2	$K_3Fe(CN)_6$	2,6-Dichlo-rophenol-indophenol
Component 1*....................................	0	0	0
" 2*..	0	22.2	29.8
" 2 + excess Component 1*..............	1092	994	719
		mg.	
" 1 required for half maximal velocity of 1 mg. of Component 2...........................	66.6	43.7	206.0

Oxygen uptake was determined in Test System A with 28.4 γ of Component 2 and 0 to 6.23 mg. of Component 1. Ferricyanide reduction was determined in Test System B with 5.7 γ of Component 2 and 0 to 1.87 mg. of Component 1. 2,6-Dichlorophenolindophenol reduction was determined in Test System C with 2.84 γ of Component 2 and 0 to 0.62 mg. of Component 1.
  * The values are given in micromoles per mg. of component 2 protein.

(10). It has been shown that neither acetyl phosphate (4) nor acetyl CoA is an intermediate of the acetate-generating system and that CoA is not required and does not function (5).

The pyridine nucleotide coenzymes do not appear to function in the *P. vulgaris* system. The addition of DPN or triphosphopyridine nucleotide (TPN) does not affect the rate of pyruvate oxidation, nor is there an increase in the optical density of the system at 340 mμ after zero oxygen tension has been reached. Additional evidence for the non-participation of DPN is the failure of the system to dismute pyruvate in the presence of added lactic dehydrogenase and DPN and the ability of crude extracts to oxidize pyruvate at an undiminished rate after the DPNH oxidase in the extract was completely inactivated by heating at 55° for 20 minutes. The non-function of lipoic acid is suggested by the fact that this coenzyme

is present in the *P. vulgaris* system at one-hundreth of the level found in acetyl CoA-generating systems. The failure of 0.03 M arsenite to inhibit the reduction of 2,6-DCP by Component 2 and oxygen uptake by both components suggests also the non-function of lipoic acid, as arsenite at lower concentrations is a potent inhibitor of lipoic acid-requiring enzymes (3). The amount of the complete *P. vulgaris* system which permits an oxygen uptake of 1200 µl. per hour contains 3.66 γ of FAD. The D-amino acid oxidase system requires 33.3 γ of FAD to achieve a similar rate. The failure of this system to reduce nitrofurans and tetrazolium salts, compounds readily reduced by flavin-linked oxidases, also suggests that FAD is not functional in this system.

TABLE IV

Diphosphothiamine Requirements for Pyruvate Oxidation or Dismutation by Proteus vulgaris

The pyruvate was metabolized in micromoles per ml. of cell-free extract.

Cell-free extract prepared from		Pyruvate metabolized per hr.	
		Oxidation	Dismutation
16 hr. cells	+DPT	238.0	2.2
	− "	5.4	2.2
6 hr. cells	+ "	64.4	30.0
	− "	22.2	25.0

Pyruvate oxidation was determined in Test System A; dismutation was determined in a system similar to that of Korkes *et al.* (16) by estimating acetyl phosphate production.

The only coenzyme common to both the *P. vulgaris* acetate-generating system and the acetyl CoA-generating system is DPT. The former system is obtained resolved for DPT in the preparation of cell-free extracts by ultrasonic oscillation (4) or by sonic oscillation (6), while the latter systems are obtained from *E. coli* and *S. faccalis* (3) containing sufficient bound DPT for maximal activity. Cell-free extracts of *P. vulgaris* contain both the acetyl CoA-generating dismutase system and the acetate-generating oxidase, making it possible to determine whether the variation in the DPT-enzyme associations is a species difference or a difference between enzyme systems. The dismutation rate in extracts prepared from 16 hour cells is less than 5 per cent of the oxidation rate. The addition of DPT causes a 40-fold stimulation of pyruvate oxidation, but it has no effect on the dismutation rate. The dismutation rate in an extract from 6 hour cells is 50 per cent of the oxidation rate. The addition of DPT to this extract causes a 3-fold stimulation of the oxidation rate and has only a small effect on the

dismutation rate (Table IV).   The difference in the nature of the DPT requirement is not due to widely varying DPT-enzyme association constants since these constants are similar when both systems are resolved for DPT.   If one DPT-requiring enzyme functioned in both acetate and acetyl CoA generation, it would be necessary that bound DPT be available to this enzyme for one kind of reaction and unavailable to that same enzyme for another kind of reaction.   A more plausible explanation of these results is the existence of two separate enzymes, one functioning in acetate generation and the other in acetyl CoA generation.   Supporting the latter interpretation are the separation of both types of enzyme systems from *E. coli* and *P. vulgaris* and the demonstration that the fractions of each type system would not replace each other (11).

DISCUSSION

The oxidation of pyruvate to free acetate by the enzyme system from *P. vulgaris* had been thought to occur through pathways differing only in minor details from the mechanism by which acetyl CoA is formed from pyruvate (6).   However, partial purification and further study of the acetate-generating system have led to the conclusion that differences between this system and the acetyl CoA-generating systems are extensive.

The reactions responsible for disposing of the 2 carbon units produced by the primary decarboxylation of pyruvate in the acetyl CoA-generating systems require, in addition to CoA, lipoic acid and DPN.   It is doubtful whether these reactions occur also in the acetate-generating pyruvate oxidase of *P. vulgaris*, as CoA, lipoic acid, and DPN do not function in this system.   The primary cleavage of pyruvate in acetyl CoA-generating systems is apparently catalyzed by an enzyme, "carboxylase," which in the presence of 2,6-DCP or ferricyanide decarboxylates pyruvate to free acetate.   Component 2 of the acetate-generating system catalyzes the same over-all reaction.   The observation that, in an extract of *P. vulgaris*, acetyl CoA generation is dependent on an enzyme with bound DPT, that acetate generation is dependent on an enzyme resolved for DPT, and that "carboxylase"- and Component 2-containing fractions are not mutually replaceable suggests that Component 2 and "carboxylase" are not the same enzyme.

The oxidation of pyruvate to free acetate by the enzyme system of *P. vulgaris* may be seen to occur in the following manner: (1) pyruvate + Component 2 + DPT → "activated complex;" (2) "activated complex" + Component 1 → acetate + CO_2 + DPT + reduced Component 1 + Component 2; or (3) "activated complex" + 2,6-DCP or ferricyanide → acetate + CO_2 + DPT + Component 2 + 2,6-$DCPH_2$ or ferrocyanide.

Pyruvate is oxidized by Component 2, which may be considered to be a

pyruvic dehydrogenase, to acetate and carbon dioxide. The electrons are slowly accepted by artificial oxidants or more rapidly by the cytochrome system of Component 1, which is in turn reoxidized by oxygen or by the artificial electron acceptors. The rapid reaction with the cytochrome system and the slower reaction with artificial oxidants have in common the failure to preserve the high energy moiety of the pyruvate molecule.

Methods and Materials

Ferrocyanide was determined by forming ferriferrocyanide (Prussian blue) on the addition of a ferric salt (10). Protein and nucleic acid were estimated from the ratio of optical densities at 280 and 260 mμ according to the calculations of Warburg and Christian (12). Metabolites were estimated by the following methods: CoA by the stimulation of bacterial transacetylase, lipoic acid by the method of Gunsalus *et al.* (13), FAD by the stimulation of D-amino acid oxidase (this assay was made by Dr. Arnold Brodie), acetate by the lanthanum nitrate method of Hutchens and Kass (14), and acetyl phosphate by the hydroxamic acid method of Lipmann and Tuttle (15). Crystalline lactic dehydrogenase was prepared according to the directions of Korkes *et al.* (16), and transacetylase was prepared from water extracts of *Clostridium butyricum* by precipitation with acetic acid at pH 4, followed by ammonium sulfate fractionation between 0.60 and 0.70 saturation.

Other chemicals used were obtained from the following sources: CoA, 75 per cent pure, from the Pabst Laboratories, DPT from Merck and Company, Inc., DPN, 80 per cent pure, from the Schwarz Laboratories, Inc., TPN, 80 per cent pure, and DPNH from the Sigma Chemical Company, and 2,6-DCP from The Matheson Company, Inc.

SUMMARY

The pyruvate oxidase system of *Proteus vulgaris* produces free acetate without the intermediate formation of acetyl coenzyme A. It consists of a particulate autoxidizable cytochrome system and of soluble pyruvic dehydrogenase. The pigments of the cytochrome system have been identified as cytochromes a_1, a_2, and b_1. The dehydrogenase has been extensively purified.

The dehydrogenase can reduce artificial electron acceptors; the cytochrome system greatly increases their rate of reduction and also enables oxygen to serve as the terminal oxidant.

Evidence is presented which indicates that coenzymes required by acetyl coenzyme A-generating pyruvate oxidases, diphosphopyridine nucleotide, coenzyme A, and lipoic acid do not function in the acetate-generating pyruvate oxidase of *P. vulgaris*. Diphosphothiamine, a coenzyme common

to all pyruvate oxidases, is required by the pyruvic dehydrogenase of *P. vulgaris*; however, this enzyme does not function in the acetyl coenzyme A-generating system.

It has been concluded that the existence of common intermediates and enzymes in both the *P. vulgaris* pyruvate oxidase system and the acetyl coenzyme A-generating systems is doubtful.

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THE METABOLISM OF HYDROCORTISONE BY KIDNEY TISSUE IN VITRO*

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Recent investigations concerning the metabolism of steroids by kidney tissue (1–6) have led to gross observations of the capacity of this tissue to alter the functional groups of the steroid molecule. Most of these studies have been concerned with the metabolism of C-19 androgenic steroids. Schneider and Horstmann (6), however, have observed the loss of the α,β-unsaturated ketone in ring A and the α-ketolic side chain of adrenocortical hormones in kidney incubations, although they did not isolate any 17-ketosteroids from the medium.

This communication describes the conversion of hydrocortisone (Kendall's Compound F) to a number of oxidation and reduction products during incubation with kidney tissue. The potential significance of these compounds in the metabolism and physiology of the adrenocortical hormones is discussed.

Methods

Fresh bovine kidneys were obtained from the abattoir approximately 30 minutes after the death of the animals. The fat and connective tissue were removed, and the tissue was ground to a mince by means of a commercial grinder. 80 gm. of this mince were introduced into each of twenty (1 liter) Erlenmeyer flasks. To the tissue in each flask were added 150 ml. of calcium-free Krebs-Ringer-phosphate buffer, 0.1 M, pH 7.4, containing 10 ml. of 0.025 M glucose, as well as 10 ml. of 0.001 M DPN and 2 ml. of 0.001 M ATP as cofactors (3, 7). Hydrocortisone was prepared for incubation as follows: A 500 mg. portion of the free compound was dissolved in 10 ml. of 95 per cent ethanol. To this solution were added 20 ml. of propylene glycol, after which the ethanol was removed by evaporation under nitrogen at 40°. This propylene glycol solution of steroid was then added to a 10 per cent kidney homogenate (prepared with buffer). To each incubation flask were added 10 ml. of this kidney homogenate

(containing 25 mg. of the hydrocortisone), after which the contents of the flasks were incubated by gentle shaking for 4 hours at 38° in an atmosphere of 95 per cent O_2-5 per cent CO_2.

Extraction

The incubation mixture was treated according to the following procedures in order to extract the non-conjugated steroid metabolites. In this study, no attempt was made to recover the conjugated steroid fractions which occurred in the incubation medium.

At the end of the incubation period 2 volumes of acetone were added to each flask, and the contents were combined and stirred for 18 hours at room temperature. The mixture was then filtered through fritted glass, the residue resuspended in fresh acetone, and the above procedures repeated. The evaporation of the acetone in the filtrates was carried out in vacuo under nitrogen at 40°, as were all subsequent solvent evaporations. The remaining aqueous solution was extracted five times with one-half its volume of chloroform, and the chloroform extract was evaporated to dryness. The residue was dissolved in absolute methanol, diluted with distilled water to a final concentration of 90 per cent methanol, and then extracted twice with a volume of petroleum ether (b.p. 40–60°) equal to 25 per cent of the total volume in order to remove extraneous lipides. The latter extract was back-washed twice with 90 per cent methanol to recover any steroids removed by the petroleum ether. The combined methanolic solutions were evaporated; the residue was dissolved in 33 per cent methanol and was dialyzed against a 40 per cent methanol solution, according to the procedure of Axelrod and Zaffaroni (8) for 8 days. The dialysate was extracted five times with chloroform and the latter reduced to dryness. The residue was then dissolved in chloroform-methanol (1:1) and divided into forty equal portions preliminary to chromatographic analysis.

Chromatography

The forty portions were chromatographed in chambers constructed essentially as described by Burton et al. (9). The one modification involved the use of a Chromatoplat,[1] which permitted the simultaneous use of two solvent troughs in each chamber instead of the conventional 1-trough stand. In effect this served to expedite the chromatographic analysis of the large number of samples.

To permit the chromatographic resolution of any C-19 and C-21 steroid metabolites present, the chromatographic solvent systems and techniques of Zaffaroni et al. (10), Axelrod (11), and Arroyave and Axelrod (12) were

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[1] Obtained from Nalge Company, Rochester, New York.

used in this study.   Fig. 1 describes schematically the detailed chromato-
graphic analysis.

Qualitative and Quantitative Analysis

The steroid components were detected on the chromatograms by means
of the following color tests and reagents: triphenyltetrazolium chloride
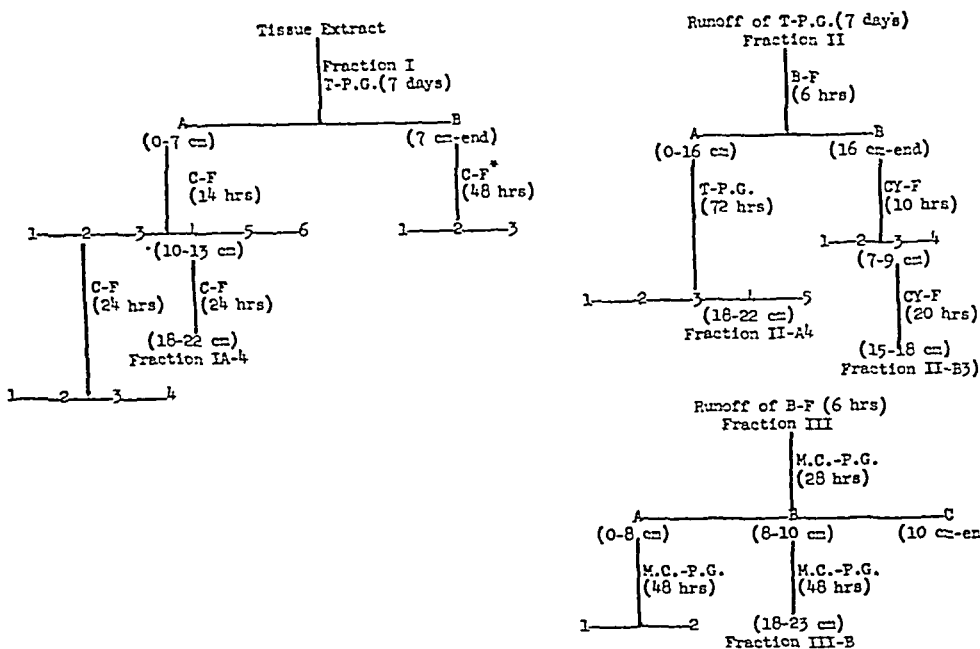
Figure 1

FIG. 1. Chromatographic details for the separation of steroid metabolites isolated
after the incubation of hydrocortisone with kidney mince. T-P.G., toluene-propyl-
ene glycol; C-F, chloroform-formamide; B-F, benzene-formamide; M.C.-P.G.,
methylcyclohexane-propylene glycol; CY-F, cyclohexene-formamide.

* The remaining free hydrocortisone was recovered in the FI-B runoff.

(TPTZ) (13), fuming sulfuric acid (15 per cent free SO_3) (14), alkaline
m-dinitrobenzene (m-DNB) (11), and acidic 2,4-dinitrophenylhydrazine
(DNPH) (11).   The differential identification of the corticoid side chains
was effected by utilizing the procedures described by Axelrod (15).   After
final chromatographic purification, portions of each compound were taken
for mixed chromatograms (16) with authentic samples[2] and for spectro-

photometric analysis with a Beckman DU spectrophotometer, utilizing the ultraviolet absorption maxima from 220 to 600 mμ in absolute methanol, in concentrated sulfuric acid (17), and in fuming sulfuric acid (18). In addition, the concentrated sulfuric acid absorption spectrum of a mixture of the compound and an authentic sample, after mixed chromatography, was obtained. The acetates of the purified C-21 compounds, prepared by the conventional pyridine-catalyzed reaction with acetic anhydride and extracted by ether, and the free C-19 compounds were subjected to infrared analysis.[3]

TABLE I

Characterization of Products of Hydrocortisone Incubation with Bovine Kidney Mince

Identified compound	Designation (see Fig. 1)	Quantity recovered, mg.	Absorption maxima, mμ		
			Concentrated sulfuric acid	Fuming sulfuric acid (15 per cent free SO$_3$)	Concentrated sulfuric acid mixture*
Δ^4-Pregnene-11β,17α,20β, 21-tetrol-3-one........	FI-A4	25.40	240, 285, 460	280, 480	240, 285, 460
Δ^4-Pregnene-17α,21-diol-3,11,20-trione.........	FII-A4	2.45	283, 340, 415	280, 430	283, 340, 415
Δ^4-Androstene-11β-ol-3,17-dione............	FII-B3	4.25	285, 380, 465	285, 475	285, 380, 465
Δ^4-Androstene-3,11,17-trione..................	FIII-B	0.83	285	280	285
Δ^4-Pregnene-11β,17α,21-triol-3,20-dione (hydrocortisone)	FI-B runoff	186†	240, 280, 395, 470		240, 280, 395, 472

* After chromatography and elution of a band consisting of a mixture of the fraction recovered from the incubation medium and a sample of the authentic compound.

† Corrected for losses due to extraction, chromatography, and elution procedures.

The quantitative estimation of each of the identified compounds was carried out on the basis of its absorption maximum at 240 mμ in absolute methanol. The comparative recovery of the identified metabolites is presented in Table I. Of the 500 mg. of hydrocortisone initially added to the incubation medium, only 186 mg. were recovered. This value includes conservative corrections for losses in extraction, in chromatography, and in elution. It is noteworthy that the quantity of steroids isolated represents minimal values, since the procedures used involve the extraction of only the non-conjugated steroid metabolites.

Results

Although twenty-five fractions were isolated from the kidney incubation, only four compounds, which were among those present in the largest quantity, have been definitely identified. Lack of sufficient material has thus far prevented complete identification of the remaining compounds. All four compounds identified had in common the α,β-unsaturated ketone group in ring A. Although some of the unidentified compounds were found to contain the reduced α,β-unsaturated ketone (*i.e.*, the "tetrahydro" form), these were among the fractions which were present in minute amounts.

The four compounds have been identified as follows.

FI-A4 (Δ^4-Pregnene-11β,17α,20β,21-tetrol-3-one)—Mixed chromatograms with pure Δ^4-pregnene-11β,17α,20β,21-tetrol-3-one in chloroform and toluene systems portrayed identical chromatographic behavior. Spot tests revealed the presence of a glycerol type side chain and an α,β-unsaturated ketone grouping. Confirmation of the presence of the α,β-unsaturated ketone group was obtained by demonstrating an absorption maximum of this compound at 240 mμ in absolute methanol. This compound and a mixture of this compound with authentic Δ^4-pregnene-11β,17α,20β,21-tetrol-3-one gave an absorption spectrum identical with that of the authentic sample in concentrated sulfuric acid (Table I). The same procedure with fuming sulfuric acid again gave an absorption spectrum identical with that of the authentic sample. Infra-red analysis confirmed the identity of this compound. In all, 25.40 mg. of this compound were isolated from the incubation.

FII-A4 (Δ^4-Pregnene-17α,21-diol-3,11,20-trione)—This compound was shown to be identical to Kendall's Compound E (Δ^4-pregnene-17α,21-diol-3,11,20-trione) by mixed chromatograms and by concentrated and fuming sulfuric acid chromogen absorption spectra (Table I). An ultraviolet absorption spectrum in absolute methanol showed a maximum at 240 mμ, confirming the DNPH test which demonstrated the presence of the Δ^4-3-ketone group. With TPTZ reagent the presence of an α-ketol type side chain was seen. More specifically, this was found to be a dihydroxyacetone side chain by the differential test of Axelrod (15). Infra-red analysis confirmed the identity of this compound as Kendall's Compound E. This incubation yielded 2.45 mg. of the compound.

FII-B3 (Δ^4-Androstene-11β-ol-3,17-dione)—Mixed chromatograms suggested that this compound was Δ^4-androstene-11β-ol-3,17-dione. A mixture of this compound and an approximately equal quantity of an authentic sample as well as the individual compound gave absorption spectra identical with that of the authentic sample in concentrated sulfuric and in fuming sulfuric acid (Table I). The compound in absolute methanol

gave a maximum at 240 mμ in the ultraviolet region, indicating the presence of a Δ^4-3-ketone grouping. A positive dinitrobenzene spot test showed the presence of the 17-ketone group. That this compound is Δ^4-androstene-11β-ol-3,17-dione was confirmed by infra-red analysis. Of this compound 4.25 mg. were obtained.

FIII-B (*Δ^4-Androstene-3,11,17-trione*)—The identity of this compound as adrenosterone (Δ^4-androstene-3,11,17-trione) was confirmed by mixed chromatograms and by its absorption spectra in the ultraviolet region in concentrated sulfuric acid and in fuming sulfuric acid (Table I). The presence of the Δ^4-3-ketone group was indicated by the DNPH reagent and by its absorption at 240 mμ in methanol. The 17-ketone group was shown to be present by the *m*-DNB test. Infra-red analysis confirmed these findings (Table I). Adrenosterone was obtained in the amount of 0.83 mg. from this incubation.

<div align="center">DISCUSSION</div>

In this study it has been observed that kidney tissue has the capacity to reduce the C-20 ketone group of hydrocortisone and to cleave oxidatively the side chain with the resultant formation of C-19 compounds, without active metabolism of the Δ^4-3-ketone group to reduction products of ring A. These findings substantiate the suggestions of Schneider and Horstmann that the metabolism of this type of steroid may involve the reduction at C-20 and the extensive enzymatic degradation of the side chain ((6) p. 637). Although these investigators were unable to detect any 17-ketosteroids in their experiments, such compounds were isolated and identified in the present investigation. However, our observations are contrary to the postulate of Dorfman and Ungar (19), who suggest that reduction of the double bond in ring A must precede splitting of the side chain in the metabolism of C-21 compounds to C-19 compounds.

The demonstrated ability of the kidney tissue to oxidize the 11β-hydroxy group to an 11-ketone group suggests that this oxidation is independent of the eventual splitting of the α-ketol side chain, since both Kendall's Compound E and adrenosterone were found. The relative quantities of the compounds obtained (Table I) support the view that adrenosterone is derived directly from Kendall's Compound E, whereas the 11β-hydroxy-Δ^4-androstene-3,17-dione is derived from hydrocortisone. Salamon and Dobriner (20) isolated 11β-hydroxy-Δ^4-androstene-3,17-dione from human urine after the administration of corticotropin (ACTH) and stated that it is highly probable that this compound is elaborated by the adrenal cortex independently of hydrocortisone synthesis. They reasoned that, "Since 11β-hydroxy-Δ^4-androstene-3,17-dione possesses in ring A the α,β-unsaturated ketone system characteristic of hormones, rather than the

saturated alcohol structure characteristic of metabolites, it seems unlikely that this substance is a metabolic transformation product of another adrenal hormone." The present experiments show that 11β-hydroxy-Δ⁴-androstene-3,17-dione may arise as a metabolic product of hydrocortisone in the kidney. The amount of this metabolite might conceivably be enhanced under conditions in which increased amounts of Compound F become available to the kidney. The fact that it has been isolated from human urine must be reckoned with in spite of the known extensive conversion of the Δ⁴-3-ketone group to the tetrahydro form in the liver (21–23).

Our observations that hydrocortisone may be metabolized to 17-ketosteroids in kidney tissue may also explain the findings of Munson (24) that the administration of hydrocortisone to orchiectomized and adrenalectomized humans yields increased 17-ketosteroid titers in their urines.

This observation, plus the fact that at least one of our products, *i.e.* 11β-hydroxy-Δ⁴-androstene-3,17-dione, has all the requisites for potent androgenic activity (20), suggests that the kidney may play a part in the refractory course of metastatic carcinoma of the prostate in adrenalectomized and orchiectomized humans (25). The renal production of small amounts of potent androgens from the maintenance doses of hydrocortisone may conceivably aggravate the prostatic malignancy.

The results described here will be extended in a forthcoming report dealing with the perfusion of kidneys with hydrocortisone.

SUMMARY

Hydrocortisone was incubated with bovine kidney tissue. The following compounds were isolated and identified: Δ⁴-pregnene-11β,17α,20β,21-tetrol-3-one, Δ⁴-pregnene-17α,21-diol-3,11,20-trione, Δ⁴-androstene-11β-ol-3,17-dione, and Δ⁴-androstene-3,11,17-trione. The significance of these findings was discussed. Included is a consideration of the renal production of potent androgens, and the rôle that such metabolites may play in the refractory course of prostatic carcinoma in orchiectomized and adrenalectomized humans maintained on adrenocortical hormones.

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D-MANNITOL 1-PHOSPHATE DEHYDROGENASE FROM ESCHERICHIA COLI*

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D-Mannitol is the most widely distributed of the naturally occurring hexahydric alcohols, and the compound has been known since the early part of the nineteenth century. It is found as a reserve material in some algae and many higher plants. In some lower fungi it is the product of the fermentation of sugars. Finally, mannitol is utilized for growth and fermented by a large number of species of microorganisms.

Whereas some fungi can convert certain aldohexoses and aldopentoses to mannitol, the latter has never been found in the medium after growth on fructose (2–4). On the other hand, several species of bacteria can form mannitol from fructose. Peterson and coworkers (5–7) studied this phenomenon in pentose-fermenting organisms, and Liu (8) and Sebek and Randles (9) observed the oxidation of mannitol to fructose in pseudomonads. No phosphorylated intermediates appeared to be involved in that conversion, as indicated by the absence of phosphorylation on anaerobic incubation of whole cells with hexitol and ATP[1] (9). It is known that the same carbohydrate may be dissimilated by different organisms either directly or only after phosphorylation. This paper describes studies of an enzyme from *Escherichia coli* which catalyzes the oxidation of man-

[1] The following abbreviations will be employed in this paper: DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; DPNase for diphosphopyridine nucleotidase; ATP for adenosine triphosphate; TPN and TPNH for oxidized and reduced triphosphopyridine nucleotide; F-6-P for fructose 6-phosphate; mannitol 1-P for mannitol 1-phosphate; sorbitol 1-P for sorbitol 1-phosphate; glucose 6-P for glucose 6-phosphate; and mannose 6-P for mannose 6-phosphate.

nitol phosphate in the presence of DPN, but will not act on the non-phosphorylated hexitol.[2]

Materials—DPN was purchased from either the Sigma Chemical Company or the Pabst Laboratories and was approximately 90 per cent pure. DPNH was prepared according to the method of Pullman *et al.* (10). The concentration and purity of solutions of oxidized and reduced nucleotides were assayed by the procedure of Racker (11) with the extinction coefficient 6.22×10^6 sq. cm. mole^{-1} (12). Deamino DPN was prepared as described by Kaplan *et al.* (13). TPN of approximately 80 per cent purity was prepared from DPN by the method of Wang, Kaplan, and Stolzenbach (14). The oxidized and reduced 3-acetylpyridine analogues of DPN were prepared according to the method of Kaplan and Ciotti (15).

The barium salts of fructose 6-phosphate, glucose 6-phosphate, and fructose 1,6-diphosphate were purchased from the Schwarz Laboratories. A sample of fructose 1-phosphate was generously supplied by Dr. G. T. Cori. We wish to thank Dr. B. L. Horecker for donations of ribulose 5-phosphate and sedoheptulose 7-phosphate, Dr. M. W. Slein for mannose 6-phosphate, Dr. H. A. Lardy for sorbose 6-phosphate and tagatose 6-phosphate, and Dr. R. D. Hotchkiss for mannitol phosphate prepared by the method of Seegmiller and Horecker (16). Larger quantities of mannose 6-phosphate were synthesized in the course of this study from mannose by the chemical method of Posternak and Rosselet (17). All the barium salts were converted to potassium salts before use by addition of an equivalent amount of K_2SO_4. Potassium borohydride for the reduction of phosphate esters was obtained from Metal Hydrides, Inc., Beverly, Massachusetts.

Crystalline yeast alcohol dehydrogenase was prepared by the method of Racker (11). Purified DPNase from *Neurospora* was prepared as the fraction precipitating with 60 per cent acetone at pH 2.7 according to the procedure of Kaplan *et al.* (18). In the preparation of lactic dehydrogenase, the directions of Kornberg and Pricer (19) were followed. Bovine intestinal alkaline phosphatase was obtained from the Armour Laboratories. Dr. L. Shuster kindly furnished samples of prostatic acid phosphomonoesterase prepared according to the procedure of Markham and Smith (20).

Methods—All spectrophotometric measurements were made with a Beck-

[2] In the course of these studies, Dr. R. D. Hotchkiss and Dr. J. Marmur informed us that a similar system had been found in mutants and transformants of *Diplococcus pneumoniae*. We wish to thank Dr. Hotchkiss and Dr. Marmur for making this information available.

man spectrophotometer, either model DU or B, with plastic cuvettes (3.0 ml. capacity) or quartz cuvettes (1.5 ml. capacity), all having a 1.00 cm. light path. In some of the colorimetric determinations a Klett-Summerson photoelectric colorimeter was employed.

Reducing sugar was determined according to the alkaline copper-arsenomolybdate method of Somogyi (21) or by the anthrone procedure later published by Mokrasch (22). Fructose and F-6-P were estimated by the method of Roe (23). Hexitols were determined by estimation of formaldehyde produced after mild periodate oxidation, according to the procedure of West and Rapoport (24), except that for the acid $SnCl_2$ reagent a more stable As_2O_3 reagent (1.125 M NaOH and 0.55 M As_2O_3) was substituted to destroy excess of periodate before addition of the chromogenic solution. Inorganic phosphorus was measured by the method of Fiske and Subbarow (25), and total phosphorus after sulfuric acid digestion of the deproteinized sample. Protein was estimated according to the procedure of Lowry et al. (26).

For paper chromatograms Whatman No. 1 or No. 4 filter paper was used. Phosphate esters were developed in the solvent systems of Bandurski and Axelrod (27), Hanes and Isherwood (28), and Mortimer (29). Sugars and hexitols were run in the developer (1-butanol (5 volumes)-glacial acetic acid (1 volume)-water (2 volumes)) of Hough (30). Phosphate ester spots were detected with the method of Burrows, Grylls, and Harrison (31), aldohexoses with the aniline hydrogen oxalate reagent of Horrocks and Manning (32), aldoketoses with the acid resorcinol spray reagent of Bryson and Mitchell (33), and hexitols with the alkaline silver reagent of Trevelyan et al. (34).
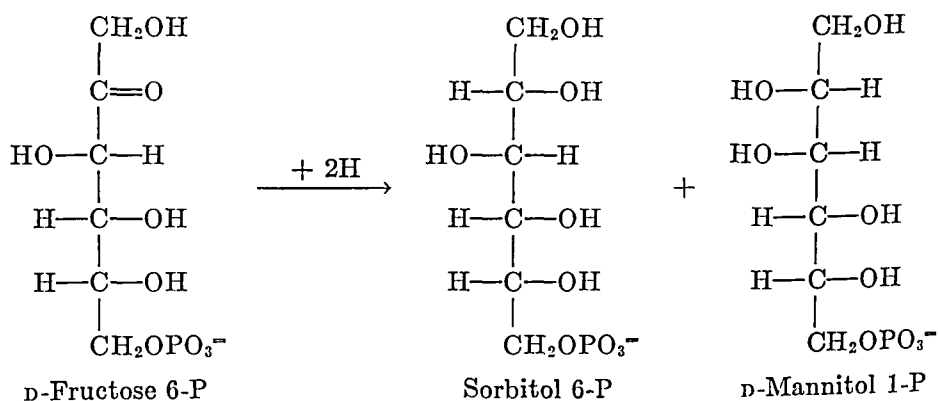
For ion exchange chromatography of sugar phosphates the procedure of Khym and Cohn (35) was followed, while the methods of Khym and Zill (36) and of Zill, Khym, and Cheniae (37) were used for sugars. It should be pointed out that the separation of fructose, sorbitol, and mannitol was accomplished independently in these studies just prior to publication of the paper last mentioned.

Growth of Bacteria—Large amounts of *E. coli* B were grown on a medium containing salts, yeast extract, and glucose. The cells were harvested by centrifugation, washed, and lyophilized. These dried cells were kept at room temperature without loss of enzymatic activity.

Aerobacter aerogenes (ATCC 8724) was grown by Dr. R. M. Burton. The cells were ground with alumina and extracted with phosphate buffer, and the extract was extensively dialyzed against distilled water. An extract of *Clostridium kluyveri* was also provided by Dr. R. M. Burton. *Lactobacillus plantarum* and *Lactobacillus brevis* (ATCC 10068) were grown by Mr. F. E. Stolzenbach. Dr. R. DeMoss kindly furnished extracts of
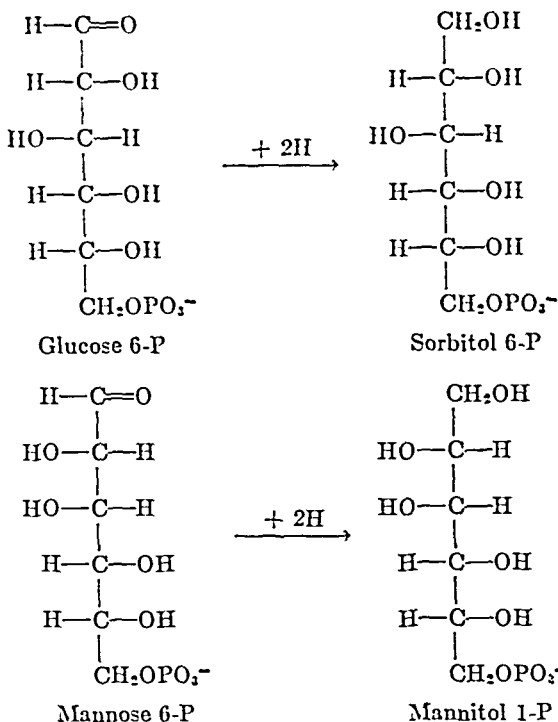
Leuconostoc mesenteroides, strain 39, grown on glucose or on arabinose, and cells of *E. coli*, Crookes strain (ATCC 8739). *Saccharomyces cerevisiae* was grown by Mr. C. DeLuca, *Pseudomonas fluorescens* (ATCC 6009-1) by Dr. H. Lenhoff, *Neurospora crassa* 5297a by Dr. D. J. D. Nicholas, and *Lactobacillus casei* (ATCC 7469 and 9595) by Dr. L. Shuster. We are grateful to all of the above colleagues for their preparations. Whenever whole cells were provided, they were suspended in 10 volumes of 0.01 M NaHCO₃ buffer and disintegrated by sonic oscillation for 10 minutes. Cell débris was removed by centrifugation, and the clear supernatant fluid was tested for activity.

Synthesis and Chemical Properties of Hexitol Phosphates—In analogy with the chemical reduction of fructose, which yields a mixture of sorbitol and mannitol, the chemical reduction of F-6-P would be expected to produce two corresponding phosphorylated hexitols. This was shown to occur and will be discussed below. When the secondary alcohol group formed from the carbonyl group of fructose on carbon atom 2 lies trans with respect to that of carbon atom 3, the compound is D-sorbitol 6-phosphate. Since carbon atoms 1 and 6 in D-mannitol have been proved to be equivalent and Rule 64 of the International Union of Chemistry concerning numbering of positions requires that "if the chain can be numbered in more than one way, the lowest numbers are given," the second diastereoisomer resulting from the chemical reduction of F-6-P should be named D-mannitol 1-phosphate.

$$
\begin{array}{cccc}
\text{CH}_2\text{OH} & & \text{CH}_2\text{OH} & \text{CH}_2\text{OH} \\
| & & | & | \\
\text{C}{=}\text{O} & & \text{H--C--OH} & \text{HO--C--H} \\
| & & | & | \\
\text{HO--C--H} & \xrightarrow{+\,2\text{H}} & \text{HO--C--H} \quad + & \text{HO--C--H} \\
| & & | & | \\
\text{H--C--OH} & & \text{H--C--OH} & \text{H--C--OH} \\
| & & | & | \\
\text{H--C--OH} & & \text{H--C--OH} & \text{H--C--OH} \\
| & & | & | \\
\text{CH}_2\text{OPO}_3^- & & \text{CH}_2\text{OPO}_3^- & \text{CH}_2\text{OPO}_3^- \\
\text{D-Fructose 6-P} & & \text{Sorbitol 6-P} & \text{D-Mannitol 1-P}
\end{array}
$$

As the reduction of a ketohexose always yields a mixture of two hexitols, it was necessary to use aldohexose phosphates for the synthesis of pure hexitol phosphates. Sorbitol 6-phosphate can be prepared from glucose 6-phosphate, and mannose 6-phosphate can be reduced to mannitol 1-phosphate. Similarly, dulcitol 1-phosphate should be obtained by reducing galactose 6-phosphate.

A solution of known concentration of hexose phosphate was added at room temperature to a solution containing one-half of the concentration

$$
\begin{array}{ccc}
\begin{array}{c}
\text{H}-\text{C}=\text{O} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{HO}-\text{C}-\text{H} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{CH}_2\text{OPO}_3^-
\end{array}
&
\xrightarrow{\ +\,2\text{H}\ }
&
\begin{array}{c}
\text{CH}_2\text{OH} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{HO}-\text{C}-\text{H} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{CH}_2\text{OPO}_3^-
\end{array} \\
\text{Glucose 6-P} & & \text{Sorbitol 6-P}
\end{array}
$$

$$
\begin{array}{ccc}
\begin{array}{c}
\text{H}-\text{C}=\text{O} \\
| \\
\text{HO}-\text{C}-\text{H} \\
| \\
\text{HO}-\text{C}-\text{H} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{CH}_2\text{OPO}_3^-
\end{array}
&
\xrightarrow{\ +\,2\text{H}\ }
&
\begin{array}{c}
\text{CH}_2\text{OH} \\
| \\
\text{HO}-\text{C}-\text{H} \\
| \\
\text{HO}-\text{C}-\text{H} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{H}-\text{C}-\text{OH} \\
| \\
\text{CH}_2\text{OPO}_3^-
\end{array} \\
\text{Mannose 6-P} & & \text{Mannitol 1-P}
\end{array}
$$

of potassium borohydride in dilute NaOH, pH 9 to 10. The course of the reaction was followed by testing for reducing sugar. At the end of the reaction the pH was lowered to about 1 by adding 1 N HCl in order to destroy excess KBH$_4$, and the solution was then neutralized by adding 1 N NaOH. A small volume of Dowex 50 resin (H$^+$ form) was added to remove excess alkali ions. After centrifugation, the supernatant fluid was freed of borate, which was found to inhibit enzymatic assays and to interfere with chromatographic experiments.

Chemical Properties of Hexitol Phosphates—Repeated attempts to separate the products of F-6-P reduction by paper chromatography or by ion exchange chromatography with either Dowex 1 (formate) and elution with 0.1 M sodium formate containing a gradually increasing concentration of formic acid or with Dowex 1 (chloride) and the eluents of Khym and Cohn (35) proved unsuccessful. This indicated a close similarity in chemical properties of the two hexitol phosphates.

The new compounds were routinely estimated by periodate oxidation by the method of West and Rapoport (24). Table I shows that the hexitol phosphates formed 1 mole of formaldehyde per mole, while the non-phosphorylated hexitols yielded 2 moles of formaldehyde, and the hexose phosphates formed only negligible amounts. However, the hexitol phos-

phates were completely inert in the tests for reducing sugar and ketohexose by which F-6-P was estimated.

The rates of chemical hydrolysis at 100° in 1 N HCl and 0.1 N NaOH of hexitol phosphates and hexose phosphates are illustrated in Fig. 1. The stability of hexitol phosphates to alkaline hydrolysis, as well as to acid hydrolysis, can be compared with the same property possessed by α-glycerol phosphate (38).

As further evidence that the hexitols were phosphorylated at the primary alcohol group, the ratio of inorganic phosphate liberated by acid phosphomonoesterase to the increased amount of formaldehyde produced after periodate oxidation was determined throughout the course of the

TABLE I

Analysis of Hexitol Phosphates by Periodate Oxidation, As Compared with Other Compounds

Compound	Moles formaldehyde formed per mole compound
Mannitol	2.0
Sorbitol	2.0
Fructose	1.0
Glucose	0.2–0.3
Mannitol 1-phosphate	1.0
Sorbitol 6-phosphate	1.0
Fructose 6-phosphate	0.05–0.10
Glucose 6-phosphate	0.05

Oxidation by the method of West and Rapoport (24) for 6 minutes at 23–25°.

enzymatic hydrolysis. From Table II it will be seen that initially each mole of hexitol phosphate gave rise to 1 mole of formaldehyde after periodate oxidation and that at the end of the enzymatic dephosphorylation each mole of hexitol produced 2 moles of formaldehyde when oxidized by periodate. The ratio of inorganic phosphate to additional formaldehyde formed remained close to 1; that is, the primary alcohol group with which the phosphate had been esterified could, after periodate oxidation, form a second mole of formaldehyde per mole of hexitol as soon as the phosphate group had been split off.

Results

Discovery of Enzyme and Assay—In the course of testing crude extracts of *E. coli* for phosphohexose isomerase activity, it was observed that the extracts catalyzed the oxidation of DPNH in the presence of F-6-P. There was a slight oxidation of DPNH in the absence of added substrate.

Ketose was found to disappear at the same time that DPNH was oxidized, indicating that F-6-P was reduced by the coenzyme in the presence of the bacterial extract. When the absorbance at 340 mμ had reached an equi-
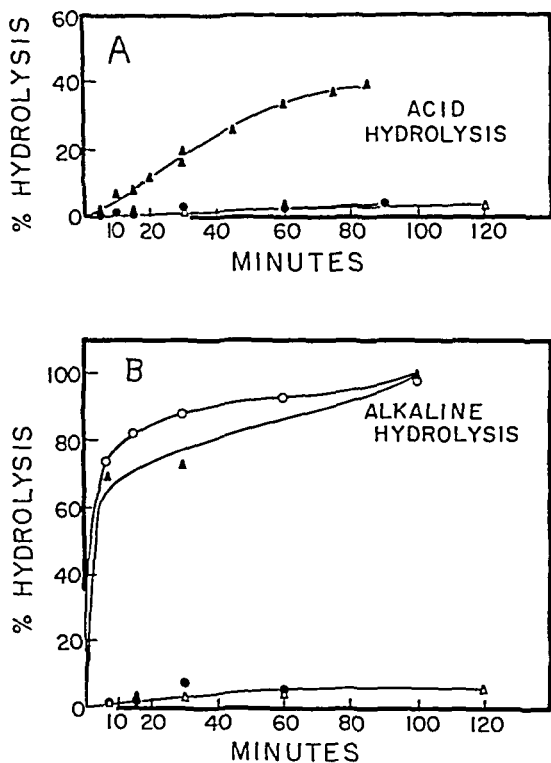

FIG. 1. Chemical hydrolysis of hexose phosphates and hexitol phosphates. *A*, acid hydrolysis. Covered tubes, containing 2.4 μmoles of ester and HCl to a final concentration of 1.0 N in a total volume of 1.0 ml., were placed in a boiling water bath. After given intervals, one tube of each series was removed and cooled, then assayed for inorganic phosphate. Control tubes with identical contents were tested at 0 minutes for total and inorganic phosphate, and the per cent hydrolysis was calculated on that basis. ▲, F-6-P; △, mannitol 1-P; ●, sorbitol 6-P. *B*, alkaline hydrolysis. Covered tubes, containing the same amount of ester as in *A* and NaOH to a final concentration of 0.1 N in a total volume of 1.0 ml., were boiled and assayed as described in *A*. ○, glucose 6-P; ▲, F-6-P; △, mannitol 1-P; ●, sorbitol 6-P.

librium value, an excess of acetaldehyde and of crystalline yeast alcohol dehydrogenase was added; the absorbance immediately rose to the value noted at the beginning of the reaction (Fig. 2). This was confirmation that one of the products of the new reaction was DPN. The reaction did not proceed without added extract or with boiled extract, and its initial rate was found to be proportional to the amount of bacterial extract

added.   The nature or pH of the buffer used appeared to have no striking effect on the rate and extent of the reduction of F-6-P, though a pH of 6 seemed slightly more favorable than a neutral or alkaline buffer.

TABLE II

Enzymatic Hydrolysis of Hexitol Phosphates Accompanied by Increase in Formaldehyde Formed by Periodate Oxidation

Time (t)	Inorganic phosphate	CH₂O	Δ CH₂O, (t — t₀)	Inorganic phosphate / Δ CH₂O
min.	*μmoles*	*μmoles*	*μmoles*	
0	0	18.6	0	
60	17.8	37.5	18.9	0.94
90	18.2	39.0	20.4	0.90
120	19.2	39.7	21.1	0.91

20 μmoles of mixed synthetic hexitol phosphates (formed by chemical reduction of F-6-P), 365 μmoles of sodium acetate buffer, pH 4.9, and 0.10 ml. of prostatic phosphomonoesterase in a total volume of 4.0 ml., incubated at 37°.
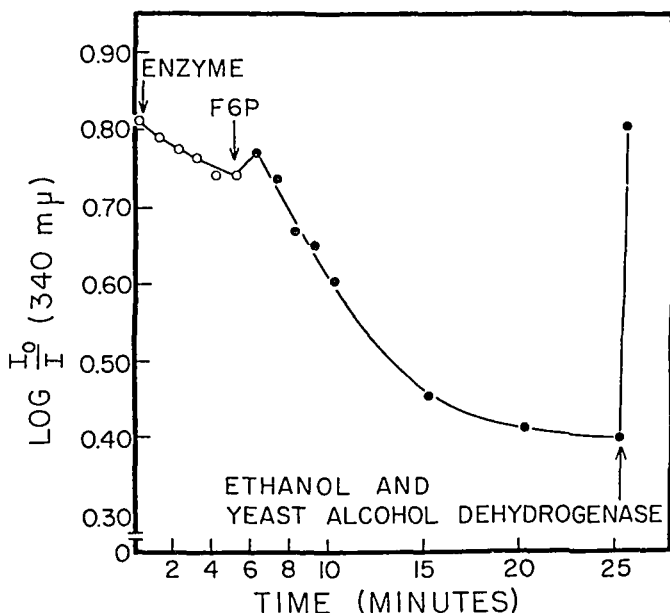

FIG. 2. Enzymatic reduction of F-6-P by cell-free extract of *E. coli*. The cuvette contained 0.2 ml. of crude *E. coli* extract and 50 μmoles of NaHCO₃ buffer, pH 8. At the second arrow 5 μmoles of F-6-P were added; at the third arrow, 100 μmoles of ethanol and 10 γ of crystalline yeast alcohol dehydrogenase. Total volume 3.0 ml., temperature 25°.

Assay Method—In a 3.0 ml. Beckman cuvette having a 1.00 cm. light path were placed 0.2 to 0.3 μmole of DPNH, 30 μmoles of potassium phosphate buffer, pH 6, 5 to 25 units of enzyme solution (for definition of

the unit see below), and water to a total volume of 2.90 ml. Absorbance was read at 340 mμ in the spectrophotometer before and at 15 to 30 second intervals after mixing with 0.1 ml. of 0.1 M F-6-P solution. 1 unit of enzyme is defined as that amount which causes an initial rate of change in absorbance (ΔA_{340}) of 0.01 per minute under the above conditions at 25°. Specific activity is expressed as units per mg. of protein. When crude bacterial extracts were assayed, it was often necessary to dilute the extract with buffer so that its absorbance at 340 mμ was small enough to permit accurate spectrophotometric readings. The rate of endogenous oxidation of DPNH must be subtracted from the rate obtained in the presence of F-6-P.

Preparation of Crude Cell-Free Extract—15 gm. of freshly harvested or lyophilized *E. coli*, strain B, were ground in a chilled mortar with an equal weight of powdered alumina (Alcoa A-301) and then extracted with 150 ml. of ice-cold 0.02 M NaHCO₃ buffer. The extract was centrifuged 10 minutes at 2300 × g at 0° and the residue of alumina and cell débris discarded. The supernatant liquid was dialyzed overnight against 4 liters of 0.02 M NaHCO₃ solution in the cold room (4°). Alternatively, the extract may be prepared by subjecting a suspension of bacteria in 10 volumes of cold buffer to sonic oscillation for 10 minutes, followed by centrifugation and dialysis.

Purification Procedure—The dialyzed crude extract was acidified to pH 4.7 with cold 1 N acetic acid. After centrifugation the supernatant fluid was neutralized and brought to 0.55 saturation (2.66 M) with solid ammonium sulfate. After centrifugation for 10 minutes at 20,000 × g (0°) the ammonium sulfate concentration of the supernatant fluid was raised to 0.65 saturation (3.16 M). After further high speed centrifugation for 10 minutes the residue containing most of the activity was dissolved in a minimal amount of water. The preparation had no detectable DPNH oxidase, alcohol dehydrogenase, lactic dehydrogenase, or α-glycerol phosphate dehydrogenase activity, but still contained some phosphohexoisomerase activity.[3] This procedure resulted in a 25- to 30-fold purification of the mannitol-phosphate dehydrogenase (see Table III).

Stability of Enzyme—The purified preparation may be kept at −20° for several months without loss of activity. It may be further dialyzed in the cold, but this is invariably attended by progressive loss of activity with time of dialysis. Thus, dialysis for 21 hours at 4° against 0.1 M NaHCO₃-0.05 M cysteine solution, pH 7, resulted in 40 to 50 per cent loss of activity. The activity could not be restored by adding boiled enzyme or 3.3 × 10⁻³ M reduced glutathione, and the loss appears to be due to protein denaturation. The enzymatic activity can be completely de-

[3] The amount of phosphohexoisomerase varied in different preparations.

stroyed by heating for 10 minutes at 60°. Heat denaturation is more rapid at pH 5 than at pH 8.

Stoichiometry of Reaction—Preliminary experiments had shown that, in order to drive the reaction as far as possible toward completion, it was necessary to use an excess of DPNH over F-6-P (2 DPNH:1 F-6-P). Furthermore, a preparation of DPNase from *N. crassa* was included in order to remove DPN as it was formed by severing its nicotinamide riboside linkage and thus draw the equilibrium further to the reduction of F-6-P. DPNase had little effect on the rate of the reaction, but increased the extent of DPNH oxidation considerably in the presence of the *E. coli* enzyme. Equilibrium was considered to have been reached when there was no further decrease in absorbance at 340 mμ. After the addition of perchloric acid to a final concentration of 10 per cent, fresh activated

TABLE III

Summary of Purification Procedure

	Total units	Total protein	Specific activity	Per cent recovery
		mg.	*units per mg. protein*	
Crude extract........................	30,800	1034	30	
Supernatant fluid, pH 4.7..............	15,600	134	117	51
Ammonium sulfate, 0.55–0.65 saturation.....	4,900	5.7	860	16

Assay conditions as described in the text.

vegetable charcoal (Pfanstiehl Chemical Company, 100 mg. per ml.) was added to the protein-free solution and the mixture allowed to stand at room temperature, with frequent shaking, for 20 to 30 minutes. The charcoal was intended to remove all oxidized and reduced DPN which would otherwise have interfered in the subsequent colorimetric determinations. About 80 per cent of the nucleotides was adsorbed in the first cycle, and the charcoal treatment was repeated as often as necessary, fresh charcoal being employed each time, to remove the nucleotides completely as judged by the lack of absorbance at 260 mμ of the supernatant fluid. The charcoal was then washed several times with water to recover any phosphate ester that had been adsorbed. The combined solution was neutralized with 5 N KOH and the resultant precipitate of KClO₄ removed by centrifugation. Representative data for the disappearance of DPNH, reducing sugar, and ketose are presented in Table IV. In spite of the variation inherent in the method of obtaining the results, there appears to be a fairly close relationship between the amount of DPNH oxidized and the amount of reducing sugar disappearing.

The reaction yields a product which, on periodate oxidation, gave a considerable increase in formaldehyde over the starting material, F-6-P. This formaldehyde is the oxidation product of a primary alcohol group which is adjacent to a secondary alcohol group. The results of Table IV indicate that for every mole of DPNH oxidized 1 mole of F-6-P is reduced, with the formation of a secondary alcohol group adjacent to an existing primary one. This suggested that the product of the reaction was apparently a hexitol phosphate.

$$F\text{-}6\text{-}P + DPNH + H^+ \rightarrow DPN^+ + \text{hexitol phosphate}$$

Isolation and Identification of Product of F-6-P Reduction—Ion exchange chromatography according to the principles developed by Khym and Cohn (35) was employed. At the end of the F-6-P reduction, an excess of both

TABLE IV

Stoichiometry of F-6-P Reduction

Δ DPNH, μmoles	Δ reducing sugar, μmoles F-6-P	Ketose disappearance, μmoles F-6-P	Increase in CH₂O after periodate oxidation, μmoles
2.3	2.2	2.5	
4.6	3.5		4.1
5.7	6.3	7.0	
2.8	3.3		

10 μmoles of F-6-P, 20 μmoles of DPNH, 0.7 to 1.3 mg. of *E. coli* enzyme, NaHCO₃ buffer, pH 8.5 to 9.0; total volume 1.5 ml.; temperature 37°; time 1 to 3 hours.

acetaldehyde and yeast alcohol dehydrogenase was added to the boiled mixture in order to oxidize any residual DPNH. The solution was aerated to remove acetaldehyde, and a preparation of DPNase from *N. crassa* was added to the mixture to split the nicotinamide portion from the adenosine diphosphoribose moiety of DPN. The reaction was followed spectrophotometrically by the cyanide procedure of Colowick *et al.* (39). After completion of this reaction, the mixture was deproteinized by boiling for 10 minutes, and the supernatant fluid was concentrated under reduced pressure. The concentrate was brought to pH 9 with NaOH solution and adsorbed on a column (0.8 sq. cm. × 9 cm.) of Dowex 1 (formate) resin when solutions of sodium formate-formic acid of decreasing pH were used as eluents, or on Dowex 1 (chloride) when the eluents of Khym and Cohn were used. All 10 to 15 ml. fractions were tested for absorbance at 260 and 340 mμ, total and inorganic phosphate, ketohexose, and periodate-oxidizable material. Nicotinamide could be washed from the column with water. The product, assumed to be a hexitol phosphate, was

eluted in the first few fractions after development of the chromatogram had begun. Inorganic phosphate followed soon afterwards. To elute F-6-P it was necessary to use 0.03 N NH$_4$Cl. Adenosine diphosphoribose was tightly bound, but could be eluted with 1 M HCl. Total recovery in terms of total phosphate was approximately 95 per cent.

In ascending paper chromatograms a new phosphate ester was detected at R_F 0.61 which gave a positive test for total carbohydrate but a negative test for reducing sugar or ketohexose.

The reaction mixture after enzymatic reduction of F-6-P was freed of pyridine nucleotide by charcoal adsorption, and the phosphate esters were dephosphorylated with intestinal or prostatic phosphatase (20). After deproteinization by boiling, a portion of the mixture was assayed
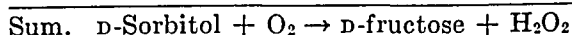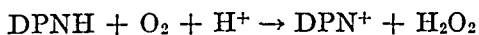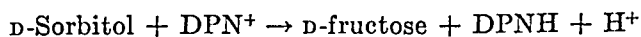
TABLE V

Assay of Sorbitol with Liver Sorbitol (L-Iditol) Dehydrogenase

The tubes contained, in 1.0 ml. total volume, potassium phosphate (pH 7.8) 350 μmoles, DPN 0.7 μmole, *C. kluyveri* extract 0.1 ml., and rat liver sorbitol dehydrogenase 2.0 mg. The values in the table represent micromoles.

Sorbitol added .... Mannitol added	0	0.1	0.25	0.5	0.66	1.0	5.0
Fructose found	0	0.07	0.24	0.45	0.59	0.90	0

Incubated at 37° for 60 minutes; trichloroacetic acid added to 5 per cent final concentration. Aliquot of filtrate analyzed for fructose by method of Roe. Fructose values corrected for a small amount of color formed in the blank by liver extract.

for sorbitol with a preparation of sorbitol (L-iditol) dehydrogenase (40) from rat or beef liver. This enzyme has been shown to oxidize sorbitol to fructose, but it will not act on mannitol or phosphorylated compounds. The results are presented in Table V. The crude extract of *C. kluyveri* containing DPNH oxidase (41) was added to shift the equilibrium of the reaction as far as possible toward the complete oxidation of sorbitol by reoxidizing DPNH as it was formed. This assay is sensitive to about 0.1 μmole per ml. of sorbitol, measured after the dehydrogenase reaction as fructose (Table V).

$$\text{D-Sorbitol} + \text{DPN}^+ \rightarrow \text{D-fructose} + \text{DPNH} + \text{H}^+$$

$$\underline{\text{DPNH} + \text{O}_2 + \text{H}^+ \rightarrow \text{DPN}^+ + \text{H}_2\text{O}_2}$$

$$\text{Sum.} \quad \text{D-Sorbitol} + \text{O}_2 \rightarrow \text{D-fructose} + \text{H}_2\text{O}_2$$

In none of the samples tested could any significant amount of sorbitol be detected, while controls containing known amounts of sorbitol gave an equivalent of fructose. This negative evidence indicated that the

product of the enzymatic reduction of F-6-P was probably not sorbitol 6-phosphate.

The hexitol could not be identified by paper chromatography, but anion exchange chromatography, according to the principles developed by Khym and Zill (36) for the separation of sugars, led to its identification. Dowex 1 (borate) in a column 0.8 sq. cm. × 13 cm. was washed with 50 ml. of 0.005 M K₂B₄O₇. 10 ml. of 0.01 M K₂B₄O₇ solution containing 10 mg. each of fructose, mannitol, and sorbitol (55 μmoles) were added, and the column was washed with 10 ml. of 0.005 M K₂B₄O₇, then eluted with 0.015 M K₂B₄O₇. Fractions (20 ml.) were tested colorimetrically for ketose and periodate-oxidizable material. As may be seen in the lower part of Fig. 3, fructose was eluted first, followed by one of the hexitols. The other hexitol could not be eluted until the borate concentration was raised to 0.030 M. Fractions of the last peak were combined, concentrated to dryness under reduced pressure, and recrystallized from pyridine. The material melted at 160–164°, and authentic mannitol similarly recrystallized melted at 164–166°. Since sorbitol melts at about 97°, the second hexitol fraction was mannitol and the first therefore sorbitol. Recovery of fructose was quantitative; of the hexitols, only 60 to 70 per cent.

The dephosphorylated reaction product was chromatographed similarly, and the result is depicted in the upper portion of Fig. 3. The major portion of hexitol could not be eluted until the borate concentration had been increased to 0.030 M. This corresponds to the behavior of mannitol. Hence it appeared that the product of the enzymatic reduction of F-6-P was indeed mannitol 1-phosphate.

Reversibility of F-6-P Reducing Reaction—When the isolated hexitol phosphate was incubated in bicarbonate buffer at pH 9 with DPN in the presence of the partially purified enzyme preparation, there was a rapid increase in absorbance at 340 mμ until a new equilibrium was reached. A simultaneous increase in ketose and reducing sugar was observed. When excess of both acetaldehyde and yeast alcohol dehydrogenase was added, the absorbance at 340 mμ fell to its initial value.

As shown in Fig. 4, synthetic sorbitol 6-phosphate was inactive in this test, but both the mixture resulting from the chemical reduction of F-6-P and authentic mannitol 1-phosphate prepared from synthetic mannose 6-phosphate possessed activity comparable to that found with the isolated product of the enzymatic reduction of F-6-P when all were tested at the same concentration. A preparation of mannitol 1-phosphate synthesized by Dr. J. Marmur and Dr. R. D. Hotchkiss by direct phosphorylation of mannitol (16) was also found to be active. Thus the product of the reaction catalyzed by the enzyme from *E. coli* is D-mannitol 1-phosphate, and the enzyme has been named D-mannitol 1-phosphate dehydrogenase.

Specificity—Table VI indicates the remarkable substrate specificity of the enzyme. Neither free fructose nor glucose is reduced, and, within the limits of the availability of sugar phosphate esters, the substrate specificity of mannitol phosphate dehydrogenase appears to be absolute. The slight activity of the preparation with glucose 6-phosphate can be ascribed to contamination of the enzyme with phosphohexoisomerase which was not completely removed during purification.[3] The specificity for the electron donor in the enzyme system is also shown.
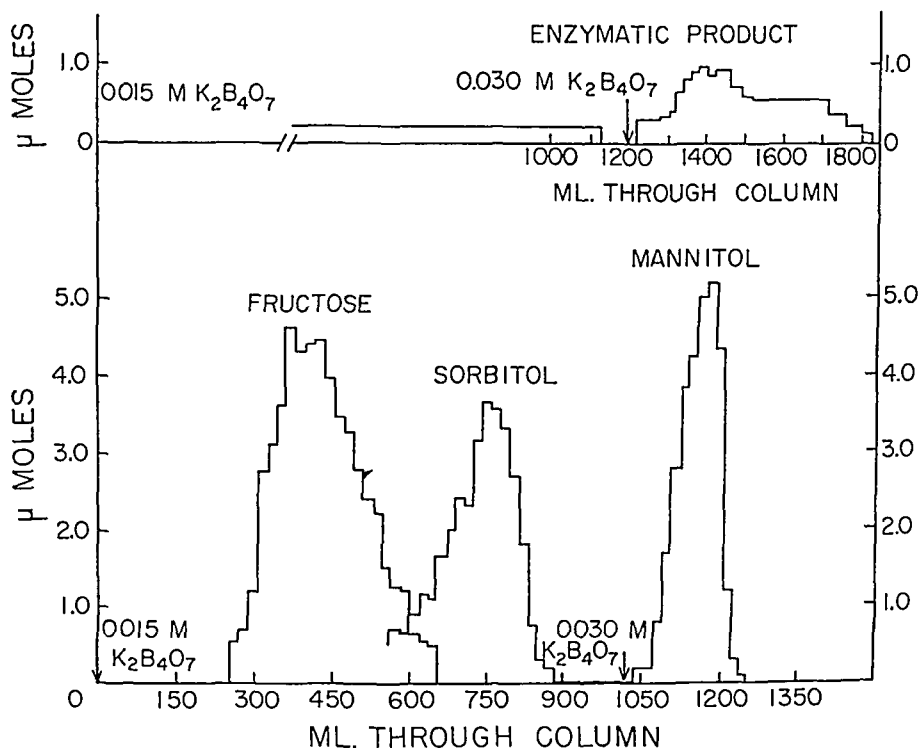
FIG. 3. Ion exchange chromatogram (Dowex 1 resin, 200 to 400 mesh, borate form) of fructose, mannitol, and sorbitol solution and of dephosphorylated enzymatic reaction product solution.

Saturation Curves—Values of the Michaelis constants for half saturation of the enzyme, calculated by the method of Lineweaver and Burk (42) and plotted by the method of least squares, are 1.17×10^{-3} M F-6-P, 1.40×10^{-3} M mannitol 1-phosphate, 2.49×10^{-4} M DPNH, and 2.29×10^{-4} M DPN.

Effect of Inhibitors on Mannitol 1-Phosphate Dehydrogenase—Semicarbazide, hydroxylamine, and sodium fluoride (all at 5×10^{-2} M) did not influence the enzymatic oxidation of mannitol phosphate. The absence of inhibition by disodium ethylenediaminetetraacetate (5.4×10^{-4} M), KCN, or NaN$_3$ (10^{-3} M), considered together with the lack of stimulation

of purified enzyme preparations by added magnesium ions (2.5×10^{-3} M), indicates that the enzyme system has no metal requirement. The 60 per cent inhibition by 10^{-4} M p-chloromercuribenzoate could be completely reversed by subsequent addition of 5×10^{-4} M glutathione. The
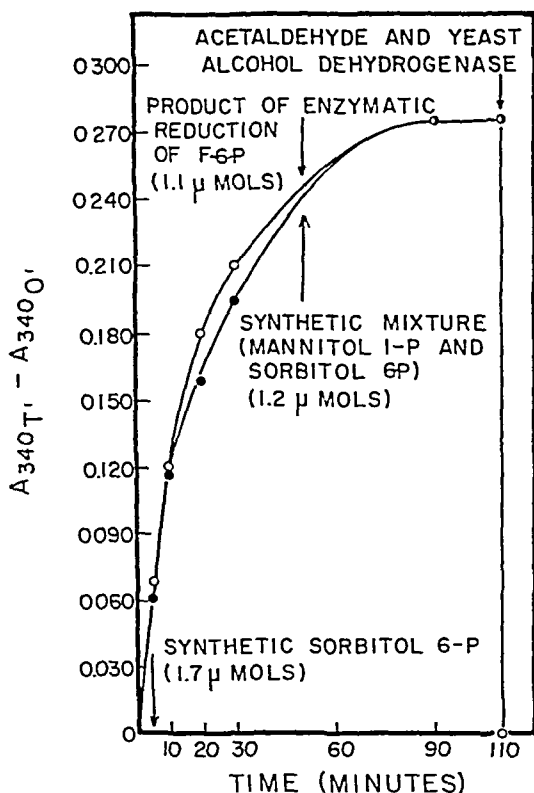

FIG. 4. Reversal of F-6-P reduction with isolated product of reaction and with synthetic hexitol phosphates. The cuvettes (1.0 cm. light path) contained 0.5 μmole of DPN, 23 units of enzyme, 1.1 to 1.7 μmoles of hexitol phosphate, and 0.1 M NaHCO₃, pH 9.3, in a total volume of 3.00 ml. At the end of the reaction 10 μmoles of acetaldehyde and 10 γ of crystalline yeast alcohol dehydrogenase were added. $A_{340\ T'} - A_{340\ 0'}$ represents the increase in optical density at a given time.

mercurial compound is generally considered to be a fairly specific reagent (at low concentration) for sulfhydryl groups. Even though *sodium iodo*acetate (5×10^{-3} M) did not inhibit the enzyme, the conclusion appears warranted that the mannitol phosphate dehydrogenase requires one or more free sulfhydryl groups for activity.

Equilibrium of Reaction—When it was found that the reduction of F-6-P was reversible, attempts were made to study the equilibrium and to

calculate an equilibrium constant for the reaction

$$\text{D-Mannitol 1-P} + \text{DPN}^+ \rightarrow \text{F-6-P} + \text{DPNH} + \text{H}^+$$
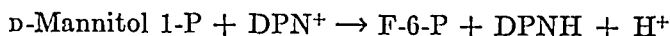
$$K_e = \frac{(\text{F-6-P})(\text{DPNH})(\text{H}^+)}{(\text{Mannitol 1-P})(\text{DPN}^+)}$$

In these experiments the relative concentrations of F-6-P and DPNH were varied, as well as the pH between 6 and 10. The values for DPN and mannitol 1-phosphate were calculated by subtracting the measured

TABLE VI

Substrate + Coenzyme Specificity of Mannitol 1-Phosphate Dehydrogenase

Substrate*	Per cent activity	Coenzyme†	Per cent activity
D-Fructose 6-phosphate	100	DPNH	100
D-Glucose 6-phosphate	10	Deamino DPNH	22
D-Fructose	0	3-Acetyl pyridine analogue of DPNH	0
D-Glucose	0	TPNH	0
D-Fructose 1-phosphate	0		
" 1,6-diphosphate	0		
D-Mannose 6-phosphate	0		
L-Sorbose 6-phosphate	0		
D-Tagatose 6-phosphate	0		
D-Ribulose 5-phosphate	0		
D-Sedoheptulose 7-phosphate	0		

* 550 μmoles of phosphate buffer, pH 7.5, 0.07 μmole of DPNH, 3 μmoles of substrate, and 15 units of partially purified enzyme, in a total volume of 1.0 ml. Temperature 23°.

† 300 μmoles of NaHCO$_3$ buffer, pH 8, 15 μmoles of F-6-P, 4 units of partially purified enzyme, 0.2 μmole of coenzyme, in a total volume of 3.0 ml. Temperature 23°.

changes in concentration of DPNH and F-6-P from the known concentrations added. Some data were also obtained, starting with known concentrations of mannitol 1-phosphate and DPN and varying the pH. Here the amount of DPN reduced was taken as equal to the F-6-P formed by oxidation of mannitol 1-phosphate. From the average K_e at pH 0 of $4.9 \pm 3.6 \times 10^{-10}$, the change in free energy for the oxidation of mannitol 1-phosphate has been calculated as $\Delta F° \approx -12,700$ calories.

Effect of pH on Enzyme Activity—As mentioned earlier, pH or the nature of the buffer (with phosphate, pyrophosphate, glycylglycine, or bicarbonate) had no marked effect on the initial rate of F-6-P reduction by the enzyme. The only effect that could be observed in numerous

attempts to detect a pH optimum for the reaction was a very gradual decline in activity from pH 6 to pH 10; therefore the lowest pH (6.0) at which DPNH is stable was generally used in assays. However, the enzymatic oxidation of mannitol 1-phosphate in the presence of DPN was found to be markedly affected by pH. As a consequence of the finding that maximal activity occurred near pH 10, NaHCO$_3$ buffer of pH 9 to 10 was used routinely in assays in this direction.

Distribution of Mannitol 1-Phosphate Dehydrogenase—Extracts of several microorganisms were tested in a mixture consisting of 2.56 μmoles of mannitol 1-phosphate, 0.60 μmole of DPN, 10 μmoles of NaHCO$_3$ buffer, pH 9.0, and 0.05 ml. of extract in a total volume of 1.0 ml. 1 unit of

TABLE VII

Distribution of Mannitol 1-Phosphate Dehydrogenase

Organism (ATCC No.)	Specific activity
	units per mg. protein
A. aerogenes (8724)	1000
E. coli (Crookes strain) (8739)	638
L. casei (9595) .	660*
" " (7469) .	350*
" plantarum	193, 110
S. cerevisiae	21
L. mesenteroides (glucose-grown)	19
" " (arabinose-grown)	0.6

All units are expressed in terms of oxidation of mannitol 1-phosphate.
* Assayed by reduction of F-6-P.

enzyme is defined as that amount which causes an initial rate of change in absorbance at 340 mμ of 0.010 per minute at 23° under the above conditions. The two *L. casei* strains were tested with F-6-P instead of mannitol 1-phosphate. Positive results are presented in Table VII. Yeast appears to resemble bacteria more than fungi in this aspect of metabolism; in the only other mold tested, *N. crassa* 5297a, no activity was detected. The high activity found in members of the colon-aerogenes and homofermentative (*L. casei*) and heterofermentative (*L. plantarum* and *L. mesenteroides*) lactobacillus groups indicates that mannitol 1-phosphate dehydrogenase represents a major pathway of mannitol and fructose utilization in those organisms.

DISCUSSION

The enzyme from *E. coli* discussed here differs in its substrate specificity from the polyol dehydrogenase of mammalian liver discovered by Blakley

(40) and further tested by Edson and McCorkindale (43, 44). The latter enzyme is believed to be identical with that from rat accessory sexual organs found by Williams-Ashman and Banks (45). Whereas the *E. coli* protein will catalyze only the reduction of F-6-P or the oxidation of mannitol 1-phosphate in the presence of pyridine nucleotide, the liver enzyme oxidizes D-sorbitol, L-iditol, and a few other sugar alcohols fitting the specificity rules deduced by Edson and McCorkindale, but it will not attack mannitol or any phosphorylated compounds. The liver enzyme, however, shares with the *E. coli* enzyme a coenzyme specificity for DPN.

The very high degree of substrate specificity and substrate affinity of the mannitol 1-phosphate dehydrogenase makes the enzyme a useful analytical tool in the study of certain phases of carbohydrate metabolism. With the partially purified enzyme preparation from *E. coli* it has been possible to detect concentrations of F-6-P as low as 2×10^{-5} M, which lies below the sensitivity of most commonly used colorimetric and chromatographic methods.

It should be noted that, although the mannitol phosphate dehydrogenase is quite specific, an enzyme capable of oxidizing sorbitol 6-phosphate has been detected in *E. coli* grown on sorbitol (46). Extracts of *E. coli* cells grown on dulcitol have been found to reduce tagatose 6-phosphate; cells grown on sorbitol or mannitol are not able to reduce this ketose sugar (46).

It is of considerable interest to note that Marmur and Hotchkiss have recently found mannitol phosphate dehydrogenase activity in *Diplococcus pneumoniae* cells able to grow and metabolize mannitol in media in which mannitol is the limiting energy source (47). This ability to utilize mannitol can be transferred to non-mannitol-utilizing cells by incubating the latter for a short time with deoxyribonucleate preparations from mannitol-utilizing mutants.

In comparing the equilibrium constant obtained in this study with values for the liver L-iditol dehydrogenase, it should be pointed out that Blakley's (40) value of 0.240 ± 0.013 at 20° and at pH 8.0 does not include the hydrogen ion concentration. On the other hand, the hydrogen ion concentration is included in the average equilibrium constant of values determined at various hydrogen ion concentrations by Williams-Ashman and Banks (45), given as 2.05×10^{-9} at 25° for the oxidation of sorbitol. These authors observed that a rise in pH displaces the equilibrium of the reaction toward the complete oxidation of sorbitol. A similar effect has been noted for mannitol 1-phosphate oxidation in the *E. coli* system. The value of the equilibrium constant obtained with the liver sorbitol system is of the same order of magnitude as that found for the *E. coli* mannitol phosphate dehydrogenase, but is about 100 times larger than those re-

ported for the oxidation of glycerol to dihydroxyacetone in *A. acrogenes*[4] or of glycerol phosphate to dihydroxyacetone phosphate by Baranowski's muscle enzyme (48). The difference may be due to the increased stability of F-6-P in its 2,5-furanose form. Although it is presumably the acyclic form of F-6-P with a free carbonyl group that is active in the enzymatic reduction, most of the product of the oxidation of 'mannitol 1-phosphate will be immediately cyclized to the more stable furanose form of F-6-P as soon as it is formed, thus displacing the equilibrium further toward the formation of the ketose than is possible in the case of 3-carbon compounds.

A more extensive survey of bacteria of the groups Lactobacteriaceae and Pseudomonadaceae should be made before definite conclusions can be drawn about the presence or absence of a pathway involving the phosphorylated mannitol as an intermediate. That this pathway exists in members of the coli-aerogenes and lactic acid bacteria has been shown, but it is not clear whether all organisms which can interconvert fructose and mannitol utilize the phosphorylated route or whether a direct oxidation may occur in some species, as has been suggested by Sebek and Randles and others.

SUMMARY

1. A soluble enzyme from *Escherichia coli* B has been found to reduce F-6-P reversibly to mannitol 1-phosphate in the presence of DPNH. The enzyme has been purified some 30-fold by isoelectric precipitation of protein impurities and fractionation with ammonium sulfate.

2. The enzyme has an absolute specificity for F-6-P and a high degree of specificity for DPNH; TPNH is inactive. The dissociation (Michaelis) constants are 1.17×10^{-3} M F-6-P, 2.49×10^{-4} M DPNH, 1.40×10^{-4} M mannitol 1-phosphate, and 2.29×10^{-4} M DPN.

3. The reaction product has been identified as mannitol 1-phosphate by (*a*) identity in enzymatic and chemical properties with synthetic mannitol 1-phosphate, (*b*) anion exchange chromatography after enzymatic dephosphorylation, and (*c*) lack of activity of the dephosphorylated compound with the sorbitol (L-iditol) dehydrogenase from liver.

4. Sorbitol 6-phosphate and mannitol 1-phosphate have been synthesized by potassium borohydride reduction of glucose 6-phosphate for the former and of mannose 6-phosphate for the latter. Mannose 6-phosphate was also prepared from mannose by chemical synthesis.

5. The enzyme requires one or more free sulfhydryl groups for activity, as shown by its sensitivity to *p*-chloromercuribenzoate and reversal of this inhibitory action by glutathione. No metal cofactors are required.

[4] Burton, R. M., Lamborg, M. R., and Kaplan, N. O., in preparation.

6. The pH for optimal mannitol 1-phosphate oxidation lies near 10, but the optimum for F-6-P reduction is not as sharply defined, being near pH 6.

7. The equilibrium of the reaction lies far toward reduction of F-6-P. The value of the constant is considerably higher than that for the formation of triose phosphate from glycerol phosphate, the difference presumably being due to formation of the furanose form of F-6-P.

8. The enzyme has been found in crude extracts of bacteria of the colon-aerogenes group, lactobacilli, and in yeast.

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RÔLE OF PHOSPHORYLCHOLINE IN ACETYLCHOLINE SYNTHESIS*

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Phosphorylcholine has been previously described as a constituent of tissues although its complete metabolic significance has not been investigated. Studies by Beznak and Chain (1) revealed that phosphorylcholine was relatively inert physiologically as compared to choline and acetylcholine. Nachmansohn and Machado (2) had originally proposed that phosphorylcholine might be an intermediate in acetylcholine synthesis, although Comline (3) was unable to demonstrate such activity in saline extracts of brain acetone powder. Also, atropine was found not to have any effect on the physiological response provoked by phosphorylcholine, suggesting that acetylcholine was not the active compound concerned (4). Recently, a compound believed to be phosphorylcholine was reported as appearing during acetylcholine synthesis by extracts of brain acetone powders (5). Wittenberg and Kornberg (6) isolated and purified from acetone powders of various tissues, including brain, an enzyme termed cholinephosphokinase, which catalyzed the formation of phosphorylcholine and adenosine diphosphate (ADP) from choline and adenosine triphosphate (ATP).

In the present investigation, phosphorylcholine was found to be more active than choline as a precursor for acetylcholine synthesis in brain extracts, and various metabolic mechanisms are considered.

EXPERIMENTAL

Enzyme—The enzyme system for acetylcholine synthesis was prepared according to the procedure of Nachmansohn and John (7). Twelve rats were decapitated and the brains dissected immediately. Cerebral cortices were separated from white matter, weighed, and the pooled material frozen by immersion in an acetone-dry ice mixture maintained slightly below $-10°$. The frozen tissue was ground with 10 ml. of acetone in a cold Potter-Elvehjem homogenizer and further ground with 300 ml. of acetone in a ball mill for 3 hours in the cold. The material was poured

into 300 ml. of acetone-dry ice mixture (below $-10°$). The precipitate that settled was removed by filtration and the last traces of acetone were removed with suction. The powder was air-dried and stored in a desiccator at $-10°$.

TABLE I

Effect of Various Factors on Activity of Phosphorylcholine As Substrate for Acetylcholine Synthesis

| | | Modifications in complete medium | | | | | Acetylcholine formed |
Choline	Phosphoryl-choline	ATP	CoA	Pyruvate	Acetaldehyde	Acetate	μmoles per gm. brain per hr.*
+	−	+	+	+	+	+	31.5
+	−	−	+	+	+	+	2.4
−	+	+	+	+	+	+	44.0
−	+	−	+	+	+	+	32.9
−	+	+	−	+	+	+	24.3
−	+	+	+	−	+	+	7.5
−	+	+	+	+	−	+	9.8
−	+	+	+	+	+	−	38.3
−	+	+	+	−	−	+	0
−	+	+	+	−	+	−	0
−	+	+	+	+	−	−	5.9
−	+	+	+	−	−	−	0

The complete system contained 0.5 ml. of 0.2 M sodium pyruvate, 0.1 ml. of 0.2 per cent cocarboxylase at pH 6.0, 0.2 ml. of 0.5 M potassium ferricyanide, 0.3 ml. of neutralized Pabst ATP (22 mg. per ml.), 0.3 ml. of reduced Pabst 90 per cent coenzyme A (1.1 mg. per ml.), 0.2 ml. of choline chloride (30.8 mg. per ml.) or 0.3 ml. of calcium phosphorylcholine chloride (10 mg. per ml.), 0.2 ml. of sodium acetate (45 mg. per ml.), 0.2 ml. of sodium fluoride (13.9 mg. per ml.), 0.2 ml. of neutralized cysteine hydrochloride (52.5 mg. per ml.), 0.3 ml. of acetaldehyde (23.6 mg. per ml.), 1 to 2 drops of tetraethyl pyrophosphate, 0.3 ml. of enzyme, 0.15 ml. of Krebs-Ringer phosphate buffer at pH 7.0, distilled water, total volume, 3.3 ml. Incubated for 90 minutes at 37°. Samples from flasks containing no enzyme or from flasks in which the reaction was stopped at zero time served as analytical controls.

* These figures obtained with one enzyme preparation which represented a pooling of twelve rat brains. Other preparations showed different absolute activities, but the same relations between experiments as illustrated in Table I.

For use, 0.5 gm. of powder was extracted with 10 ml. of 0.007 M potassium phosphate buffer in 0.05 M KCl, pH 7.0 to 7.2. This was accomplished by grinding the material with sand and 5 ml. of the buffer, followed by transfer with an additional 5 ml. to a cold Potter-Elvehjem homogenizer in which the material was further ground. After centrifugation in the cold, the supernatant solution was used as the enzyme source for the experiments recorded.

Incubation Medium—An incubation medium (8), modified according to the specifications of Jagannathan and Schweet (9) to favor pyruvate oxidation, was used. The complete medium contained sodium pyruvate, cocarboxylase, ferricyanide, ATP, coenzyme A (CoA), choline, acetate, fluoride, cysteine, and tetraethyl pyrophosphate.[1] Acetaldehyde and phosphorylcholine[2] were added in some experiments, and the exact additions are shown in Table I. Solutions omitted from the medium were replaced by distilled water.

General Procedure—Incubations were carried out in Warburg flasks at 37° for 90 minutes. Enzyme and cysteine solutions were placed in the side arms of the flasks. 1.0 ml. of the incubation mixture was removed for assay of acetylcholine by the procedure of Hestrin (11).

Results

Table I illustrates that, under the conditions employed, acetylcholine synthesis proceeds at a more rapid rate with phosphorylcholine as substrate than with choline.

As illustrated in Table I, in experiments in which phosphorylcholine was used the omission of ATP from the medium caused a partial decrease in acetylcholine synthesis. Omission of coenzyme A caused a considerably diminished synthesis. Acetate, pyruvate, or acetaldehyde as sole acetyl donors did not support acetylcholine synthesis to a significant extent, while the combination of acetaldehyde and pyruvate was very effective in promoting synthesis. Dialysis of the enzyme preparation was found to cause some loss in its activity, but relationships similar to those recorded in Table I were still obtained.

DISCUSSION

Phosphorylcholine appeared in these experiments to be a more effective substrate for acetylcholine synthesis than choline in equivalent systems. According to the suggestion of Riley (12), the phosphorylcholine might supply an energy-rich compound to the system. The mechanism suggested would necessitate an oxidation of the choline portion of the molecule, which seems unlikely in this system, since added phosphorylcholine was the sole source of the choline portion of the acetylcholine formed. The possibility also existed that there might be a direct reaction between phosphorylcholine and acetate, acetaldehyde, or pyruvate. Actually none of these acetyl donors supported acetylcholine synthesis when used

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[1] Tetraethyl pyrophosphate was obtained from the Army Chemical Center, Maryland.

[2] Calcium phosphorylcholine was provided by Dr. Richard F. Riley, University of California, Atomic Energy Project, Los Angeles, California, and was the product prepared as described by him (10).

alone or in combination, except in the case of pyruvate plus acetaldehyde, which was very effective and for which no simple explanation is readily available. The apparent requirement for coenzyme A indicates the formation of acetyl coenzyme A prior to acetylcholine formation, rather than a direct reaction of an acetyl donor with phosphorylcholine.

The most likely hypothesis to account for the facts is the reversal of the reaction forming phosphorylcholine from choline and ATP as catalyzed by cholinephosphokinase. Thus phosphorylcholine would provide the ATP necessary for acetylcholine synthesis, and the experimental data demonstrate that phosphorylcholine is even more efficient than addition of choline and ATP.

SUMMARY

1. Phosphorylcholine appeared to be a more effective substrate than choline for acetylcholine synthesis in extracts of rat brain acetone powders with added ATP.

2. Omission of ATP nearly abolished acetylcholine synthesis when choline was employed as substrate, but caused only a small depression in synthesis when phosphorylcholine was used. Omission of coenzyme A caused a reduction in the acetylcholine formed when phosphorylcholine was used as the substrate.

3. Various mechanisms are discussed for the possible rôle of phosphorylcholine in acetylcholine synthesis.

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THE UNIENZYMATIC NATURE OF YEAST POLYGALACTURONASE*

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Yeast polygalacturonase (YPG) catalyzes the hydrolysis of pectic acid to a mixture of digalacturonic and galacturonic acids (1). The reaction course consists of (*a*) an initial rapid attack on the linear chain by a random mechanism until about 25 per cent hydrolysis has occurred, (*b*) a slower phase during which an additional 25 per cent of the available bonds are broken, and (*c*) an extremely slow hydrolysis which ceases at the 70 per cent value. The reactions are as follows:

(*a*)   Pectic acid → tetra- + tri- + di- + galacturonic acids

(*b*)   Tetragalacturonic acid → tri- + galacturonic acids

(*c*)   Trigalacturonic acid → di- + galacturonic acids

The initial hydrolysis is optimal at pH 4.4, while breakdown of the tetramer has its optimum at pH 3.5. The last reaction also proceeds faster at the lower pH.

In view of the various reaction rates, the different optimal pH values, and the presence of many substrates of widely different molecular weights, it was of interest to determine whether the YPG system consists of one or more pectic enzymes responsible for the above reactions. It should be noted that Dingle *et al.* (2) and Ayres *et al.* (3) have postulated that polygalacturonase (PG) of *Aspergillus foetidus* is a complex of at least three enzymes. Schubert (4) has reported the presence of four pectic enzymes in the culture fluid of *Aspergillus niger*. Saito *et al.* (5), on the other hand, have reported two polygalacturonases in shaking cultures of *A. niger*. It will be shown that our results give proof that YPG consists of a single enzyme, catalyzing the reactions (*a*), (*b*), and (*c*).

Methods

YPG Preparation—The enzyme solution was the culture fluid of *Saccharomyces fragilis*, obtained under growing conditions described earlier (6).

* A preliminary communication of this work was published (21).

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Determination of Reaction Rates—When pectate was the substrate, a modified Willstätter-Schudel hypoiodite method was used (7), the liberated reducing groups being expressed in millimoles. Breakdown of the oligouronides was followed by the Yemm semimicro iodometric procedure (8). Assay conditions, activity units, and the methods of substrate preparation were the same as those described in a previous paper (1). In the development of the adsorption procedure, a rapid viscometric procedure was used. A modified Wichmann pectic acid, which was fairly stable for a number of days when stored at 5° with toluene, was the substrate. It was prepared by dissolving 10 gm. of sodium polypectate (California Fruit Growers Exchange, Ontario, California) in 845 ml. of distilled water, followed by homogenization in a Waring blendor for a few minutes. 55 ml. of HCl (3.4 N) were added dropwise with vigorous stirring, producing a firm gel. After adding 100 ml. of water, the solution was again blended, boiled for 5 minutes, and filtered by suction while hot. The precipitate was immediately washed with hot water until the washings were free of chloride ions. It was then added to 600 ml. of water, adjusted to pH 5 with NaOH, and filtered. Fresh solutions were prepared from the gel every few days. For activity determinations, 9.9 ml. were pipetted into an Ostwald-Cannon-Fenske capillary viscometer (9), followed by 0.1 ml. of suitably diluted enzyme solution (added after reaching temperature equilibrium). Initial flow time was obtained by substituting buffer for enzyme. Readings were taken at short intervals and the time for 50 per cent viscosity reduction was noted. Fig. 1 shows the linear relationship between enzyme concentration and the reciprocal of the time required to reach 50 per cent viscosity.

Electrophoresis—The electrophoretic analysis of YPG was conducted in a portable Tiselius electrophoresis apparatus manufactured by The Perkin-Elmer Corporation, Norwalk, Connecticut (model 38). 2 ml. cells were used in an open system.

Determination of Protein Concentration—Two methods were used. For very small amounts of protein, the method of Lowry *et al.* (10) was employed. The protein concentrations were read from a standard curve, previously constructed with crystallized bovine plasma albumin (Armour and Company, Chicago, Illinois). Standards were freshly prepared with each series of determinations. The method could not be used when $(NH_4)_2SO_4$ was present in the solution, owing to precipitation of the Folin-Ciocalteu reagent. When this was the case, the absorption at 280 mμ (11) was used. All the solutions studied had ratios of optical densities at 280 and 260 mμ which were over 1.5, indicating little or no interference from nucleic acids. 3 ml. silica cuvettes were used in a Beckman DU quartz spectrophotometer at room temperature. Standard curves were prepared with the albumin.

Preparation of Pectic Acid Gel for Adsorption Studies—The gel was pre-
pared in the same manner as the pectic acid used for viscometric assays,
except that the final step, *i.e.* adjustment of the pH to 5, was omitted.
The material was left in the gel form and made into a slurry by blending
for a few minutes. The entire adsorption and elution procedure was done
at 4°.

Results

Concentration of YPG—Preliminary experiments showed that the enzyme
could be adsorbed almost completely from solution at pH 3 at low tem-
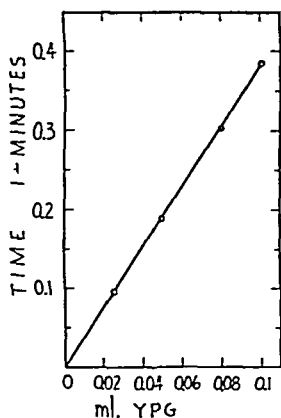
FIG. 1.   FIG. 2.

FIG. 1. Relationship between YPG concentration and the reciprocal of the time
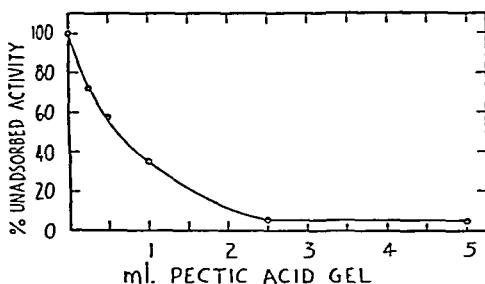required to lower the viscosity of a sodium pectate solution 50 per cent.

FIG. 2. Relationship between concentration of pectic acid gel and the amount of
YPG adsorbed. Increasing amounts of gel were added to 25 ml. samples of culture
fluid in separate beakers. The total volume was made to 30 ml. with water. The
mixtures were stirred mechanically for 2 minutes and centrifuged. The supernatant
solutions were assayed viscometrically.

peratures by pectic acid, calcium pectate, and calcium phosphate gels.
The enzyme could be eluted easily with buffers at pH 5. Pectic acid gel
was selected for further work.

In a small scale experiment, it was found that stirring the crude culture
liquid (containing 10^{-2} polygalacturonase unit per ml.) with the pectic
acid gel in a 10:1 ratio resulted in maximal adsorption (Fig. 2). A slight
foaming during the stirring procedure did not inactivate YPG. Enzy-
matic breakdown of the gel did not occur noticeably even over a period of
several hours.

For large scale work, 5.8 liters of crude YPG and 580 ml. of gel were
mixed. After centrifugation, the gel was washed with 150 ml. of 0.01 M
acetic acid (pH 3.5) for 15 minutes. This step washed out most of the

unadsorbed material but removed no YPG. Centrifugation followed, and the adsorbed protein was eluted with two successive 150 ml. portions of 1 M acetate buffer at pH 5.0. Centrifugation followed each elution and the eluates were combined and placed at room temperature overnight to decompose any pectic acid which might have dissolved during the elution.

Table I shows the results of the operation. Less YPG was adsorbed than in the small scale experiment. However, 98 per cent of the adsorbed activity was recovered in the eluate, resulting in a 13-fold concentration of activity. There was practically no increase in specific activity. There-

TABLE I

Changes Occurring during YPG Concentration on Pectic Acid Gel

Sample	Volume	Activity*	Total activity	Per cent activity	Protein† concentration after dialysis	Specific† activity
	ml.	*per ml.*			*mg. per ml.*	*per mg. protein*
Culture fluid..........	5800	0.00935	54.23	100	0.059	0.165
Supernatant after adsorption..............	6270	0.00140	9.41	17.3		
Washings..............	200	0.00004	0.01	<0.1		
Eluates..............	365	0.12000	43.80	80.8	0.890	0.168

* YPG determinations by hypoiodite method. 1 unit of hydrolytic activity is the amount that will release 1 mmole of reducing groups per minute at pH 5.0 from a 0.5 per cent pectate solution.

† Dialysis was necessary for specific activity determinations to remove any interfering aromatic amino acids which might still be present from the original culture medium.

fore, the following data obtained with concentrated YPG would also apply to untreated YPG.

Electrophoretic Analysis of Concentrated YPG—Ammonium sulfate was added to concentrated YPG until saturated and the precipitated protein was recovered by filtration. The precipitate was taken up in a small amount of acetate buffer (0.1 M, pH 4.0) and dialyzed in the cold against the same buffer. Electrophoresis of a 1 per cent protein solution was conducted in the same buffer. Photographs of the ascending and descending limbs were taken at various times by the modified Longsworth scanning method (12). Separation of the components did not occur well in the descending limb, resulting in one broad peak. Therefore, only ascending photographs are shown in Fig. 3. Two rapidly moving peaks are evident, the faster one comprising roughly 95 per cent of the total protein. The broad, slowly moving peak near the origin is the protein concentration

gradient anomaly (δ anomaly) and does not correspond to an additional protein component (13).

At least two proteins appear to be present in the concentrated YPG. Electrophoresis of a sample of culture fluid which had been concentrated solely by ammonium sulfate precipitation gave a similar pattern, confirming that the adsorption procedure does not change the protein composition noticeably.

Variable Solvent Solubility Test of Concentrated YPG—Since electrophoresis gives information only as to the minimal number of protein components, solubility studies were also made.
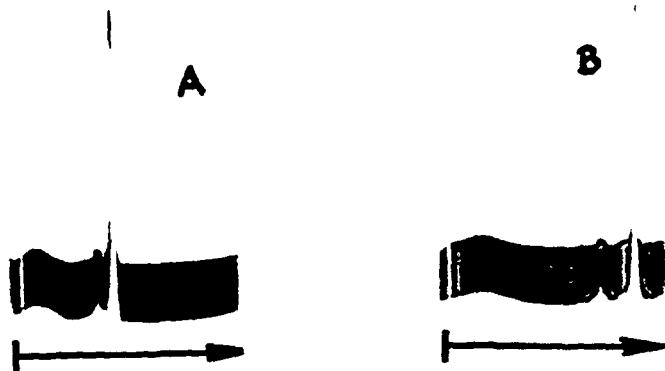
Fig. 3. Ascending electrophoretic patterns of concentrated YPG. Acetate buffer, 0.1 M, pH 4.0, μ 0.02. 132 volts; 2 ma. Temperature 1.5°. *A*, time = 4010 seconds; *B*, time = 8120 seconds. The tails of the arrows represent starting points.

In the variable solvent solubility test (14, 15), increasing amounts of ammonium sulfate are added to a constant volume of protein solution at a constant pH and temperature. After equilibrium has been reached, the precipitated protein is removed from each sample, and the protein concentration of the remaining solution is determined.

Preliminary tests showed that filter paper was unsatisfactory for separation of the precipitated protein from the solution. Whatman No. 42 paper, usually recommended for solubility studies, was found to adsorb soluble protein from ammonium sulfate solutions. The same results were obtained with Whatman No. 5 paper. Furthermore, these papers add ultraviolet-absorbing material to filtrates, complicating protein determinations. Because of these complications, centrifugation in the cold with a high speed Servall SS-1 centrifuge at 20,000 $\times$ g for 20 minutes was used.

The details are as follows: After dialyzing the concentrated YPG against 0.1 M acetate buffer (pH 5.0), increasing amounts of solid $(NH_4)_2SO_4$ were added to a series of carefully washed tubes.   15 ml. of the enzyme solution were added to each and the tubes were placed at 4° for 18 hours.   The tubes were gently agitated several times.   After centrifugation, the protein concentrations of the supernatant solutions were estimated at 280 mμ. The results were corrected for dilution caused by the presence of the ammonium sulfate (14).   The results, presented in Fig. 4, indicate two frac-
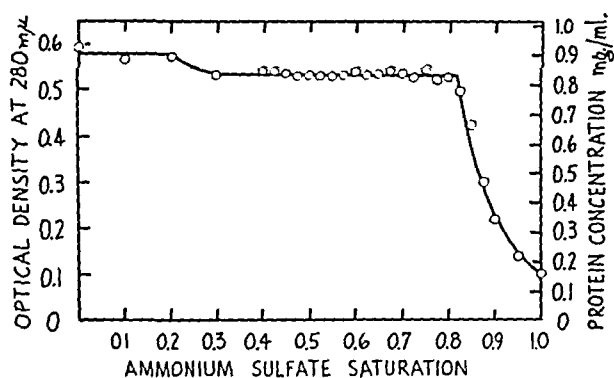

FIG. 4

FIG. 5

FIG. 4. Variable solvent solubility test of YPG concentrated by adsorption on pectic acid gel.

FIG. 5. Specific property solubility test of concentrated YPG.   The points represent the solutions used in Fig. 4, but after dialysis.   Dialysis was necessary since $(NH_4)_2SO_4$ interferes with enzyme activity.   Correction was made for further dilution caused by dialysis.
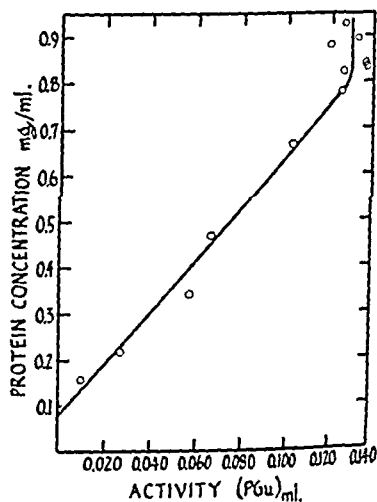
tions.   The minor fraction starts to precipitate near 0.2 saturation, while the major component is salted out between 0.8 and complete saturation.

Specific Property Solubility Test of Concentrated YPG—The specific property solubility test (16) is an extension of the variable solvent test and is conducted with the same solutions.   Its main purpose is to determine which of the protein components has the enzymatic activity.   Activities were estimated with pectic acid as substrate and plotted against the protein concentration, all with the proper corrections for dilution (Fig. 5). If one of the fractions represents pure YPG, a linear relation should be found between protein concentration and enzyme activity.   The presence of inert impurities is indicated by vertical sections representing the salting out of components having no enzyme activity.   Fig. 5 indicates that the breakdown of pectic acid is carried out by the major component.   Al-

though the points on the upper portion of the curve (where the minor component precipitates out) do not lie directly on a vertical line, the random distribution of these points and the lack of any general trend toward en-

TABLE II

Hydrolysis of Three Uronide Substrates by YPG Solutions from Various Parts of Salting Out Curve

Saturation of sample	Activities, (PGu) per ml.*			Ratios of activities		
	Pectic acid × 10 (A)	Tetragalac-turonic acid × 10² (B)	Trigalac-turonic acid × 10² (C)	$\frac{(B)}{(A)}$	$\frac{(C)}{(B)}$	$\frac{(C)}{(A)}$
0	1.26	4.95	8.72	0.039	0.018	0.00069
0.300	1.37	4.89	8.86	0.036	0.018	0.00065
0.700	1.36	5.71	9.55	0.042	0.017	0.00070
0.875	0.66	2.46	4.85	0.037	0.020	0.00073

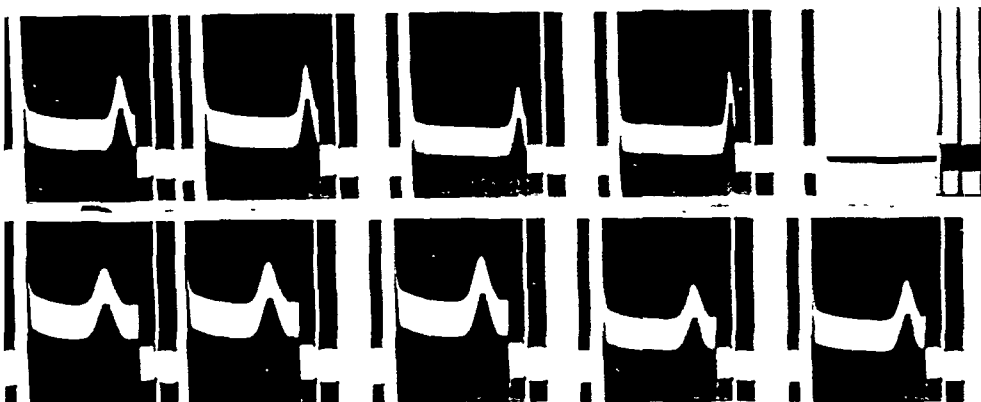* See asterisk foot-note in Table 1.

Fig. 6. Ultracentrifuge pattern of YPG in phosphate buffer, pH 6.3; μ 0.21 at 59,780 r.p.m. The photographs were taken at 8 minute intervals.

zyme loss imply that this less soluble component does not degrade pectic acid.

The slope of the YPG component enables us to predict that the specific activity of pure YPG would be 0.179 (PGu) per mg. of protein. The reason for the curve failing to go through the origin is not known.

Unienzymatic Nature of YPG—Since the major component possesses the pectic acid-degrading activity, it was important to determine whether the same enzyme is also responsible for the activity on tetra- and trigalacturonic

acids.   To do this, four of the dialyzed solutions from the specific property solubility test were allowed to act on these two uronides.   The results are shown in Table II.   On all these substrates, the greatest drop in activity occured between 0.7 and 0.875 saturation, corresponding to the salting out of the major component.   Furthermore, a fairly constant ratio among the activities on the three substrates was found, demonstrating that the hydrolysis of pectic acid and tetra- and trigalacturonic acids is caused by a single enzyme.

Ultracentrifugation—The material used for analysis in the ultracentrifuge was that which precipitated between 0.45 and full saturation.   Fig. 6 shows that the protein moved as a single boundary.   The sedimentation constant was about 4.1 at 20°, indicating a rather small molecular weight (60,000 to 100,000, depending on the shape of the molecule and other information which is still lacking).   Another run was made in acetate buffer, pH 4.0, μ 0.2, also indicating a single boundary.

<div align="center">DISCUSSION</div>

It is of interest to compare yeast polygalacturonase with other pectic enzyme preparations described in the literature.   Our results have shown that *S. fragilis* in standing cultures in a synthetic medium appears to produce a single extracellular pectic enzyme from glucose as carbon source. This enzyme, constituting about 95 per cent of the protein in the medium, catalyzes a glycosidic hydrolysis of pectic acid to a mixture of digalacturonic acid and galacturonic acid (1).   The British group (2, 3), using *A. foetidus*, found that two pectic enzymes are produced when grown in a submerged culture.   Enzyme Ia can hydrolyze pectin or pectic acid to "intermediate polyuronides" (optimum pH 5.3) and Enzyme Ib catalyzes the breakdown of pectic acid to a mixture of tri-, di-, and galacturonic acids (optimum pH 3.5).   They claim to have obtained Enzyme Ib free from Enzyme Ia by inactivating the latter at 60° for 30 minutes.   Enzyme Ib may be similar to YPG, although the former's optimal pH is lower for the initial part of the reaction (*cf.* (1)) and trigalacturonic acid is an end-product.   This last claim, however, is not entirely convincing.   The authors presented a graph showing the action of their enzyme on pectic acid (3).   When the experiment was terminated at 150 hours, galacturonic acid was still being produced.   Considering the fact that YPG attacks pectic acid 1500 times as rapidly as it hydrolyzes the trimer, it is possible that the time was not long enough for the trimer to disappear.   Saito (17), working with submerged cultures of *A. niger*, also found a complex of various pectic enzymes.   A "depolymeric polygalacturonase" (DPG) is formed as a constitutive enzyme by the fungus, accompanied by greater or smaller quantities of other pectic enzymes, depending on the presence of certain inducers.   The depolymeric polygalacturonase was separated

from the other enzymes by adsorption on filter paper and the properties reported appear to be quite similar to those of YPG, except that the end-products were found to be tri-, di-, and galacturonic acids. However, in a recent personal communication, Saito indicated that his DPG is probably identical with YPG and that the finding of trigalacturonic acid as an end-product may have been based on its very slow hydrolysis. Finally, the β-PG found by Schubert (18) in a mixture of various fungal pectic enzymes and a polygalacturonase I observed by Ozawa (19) in a mycelial extract of *Rhizopus tritici* also appear to resemble YPG, although these two enzyme preparations have not been studied in detail.

In spite of the finding that most preparations of fungal pectic enzymes are mixtures of polygalacturonases with distinctly different properties, claims that "polygalacturonase," in the more general sense, consists of various components working together to convert pectic acid to the end-product monogalacturonic acid seem to be rather speculative. In this connection, McCready and Seegmiller (20) showed that drastic purification of Pectinol 100D failed to change the ratio of its activities on polygalacturonic and digalacturonic acids. Furthermore, the evidence obtained with YPG shows that at least the breakdown of pectic acid to digalacturonic acid can be accomplished by a single protein.

SUMMARY

The investigations have shown that *Saccharomyces fragilis*, when grown in a synthetic medium with glucose as the carbon source, secretes a single pectic enzyme in nearly pure form into the culture liquid. Yeast polygalacturonase was concentrated about 13-fold by adsorption in the cold on pectic acid gel, followed by elution with 1 M acetate buffer at pH 5.0. Electrophoretic analysis and the variable solvent solubility test of concentrated YPG showed that a minor fraction (5 per cent, salting out near 0.2 saturation with $(NH_4)_2SO_4$) and a major fraction (95 per cent, salting out between 0.8 and complete saturation) were present. A specific property solubility test showed that only the major fraction had polygalacturonase activity. The ratios of the activities of various supernatant fractions on pectic acid and tetra- and trigalacturonic acids were constant after salting out, indicating that a single enzyme is responsible for the breakdown of pectic acid to digalacturonic acid. The specific activity of pure YPG was estimated to be 0.179 (PGu) per mg. of protein. Ultracentrifugation showed that the major fraction moved as a single boundary. The relation of YPG to other pectic enzymes has been discussed.

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THE TURNOVER OF LIVER GLYCOGEN IN OBESE HYPERGLYCEMIC MICE*

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The status of our information on glycogen turnover has been succinctly evaluated by Russell as follows: "At present, data on the rates of glycogen turnover are few and unsatisfactory, even for the tissues of normal animals. Most of our conclusions about regulation of processes concerned in this turnover must then be inferred from changes in the quantities of glycogen present in the tissues in different circumstances" (1). This situation is all the more surprising since the rate of turnover of liver glycogen is obviously a major characteristic of carbohydrate metabolism in any type of animal. Actually, only one attempt at determining directly the turnover rate of hepatic glycogen could be found in the literature. This attempt, performed by Stetten and Boxer (2) in 1944, was made on rats whose body fluids had been enriched with deuterium. Only six animals were used. A value of 1 day was obtained for the half life of liver glycogen.

In a recent study Stetten and Stetten (3) attempted to follow the regeneration of glycogen in the intact rat by measuring the decay of C^{14}-labeled glycogen and the proportion of radioactivity contained in the peripheral and inner tiers. They were able to demonstrate that glycogen is metabolically as well as structurally inhomogeneous, a fact which could be predicted on theoretical grounds from the results of Cori and coworkers (4–6). Regeneration of glycogen takes place more rapidly in the peripheral tiers than in the central core, a phenomenon probably ascribable to the tree-like structure of glycogen (7) and the dependence of this regeneration on the relative rates of three enzymatic reactions. However, the data of Stetten and Stetten comprised so few animals, and the absolute

amount of counts retained, as well as the specific activity of the glycogen, varied so erratically during the decay period that a calculation from their results of the rate of turnover of liver glycogen during that period is impossible.

The obese hyperglycemic syndrome is due to the mutation of a single homozygous recessive gene and the expected ratio of non-obese to obese mice of 3:1 is observed. It arose in the V stock house mouse of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, in the summer of 1949 (8). In addition to hyperglycemia and obesity these mice are characterized by increased lipogenesis even during fasting (9), hyperphagia (10), hypercholesterolemia (11), and hyperplasia of the pancreatic islands of Langerhans with greatly increased amounts of depot insulin (12). The hyperglycemia of these mice appears to be the most fundamental disturbance, since such major disturbances as increased insulin production and increased lipogenesis are explicable in terms of it.

It was of interest in the analysis of the nature of the hyperglycemia to determine the rate of hepatic glycogen turnover in the obese animals as well as their controls. The results concerning the non-obese animals would of themselves be of interest in view of the paucity of such data. To facilitate the interpretation of glycogen amounts and concentration, some data were also included pertaining to gold thioglucose-obese mice (13), a form of obesity which can be induced in Swiss mice or in thin litter mates of genetically obese animals and which is not accompanied by hyperglycemia (14) or by abnormal lipogenesis (9). The procedure consisted of following the incorporation of uniformly labeled C^{14}-glucose into glycogen. It was felt that at least on a comparative basis such a procedure was legitimate, especially in view of the facts that blood volume, total body water (15), and oxygen consumption (16) are of a similar order of magnitude in obese and non-obese animals. To base the study on decay rather than on incorporation not only would have necessitated much larger doses of the expensive glucose-C^{14}, but also would have been complicated by resynthesis of glycogen from the released glucose. It must be noted, however, that because turnover was studied under conditions of incorporation while the blood concentration of labeled glucose was declining the usual methods of calculation of rates had to be modified.

EXPERIMENTAL

Fifty-one mice, twelve female and thirteen male obese hyperglycemic and twelve female and fourteen male non-obese litter mates, which were maintained prior to and during the experimental periods on commercial laboratory chow, were used in these studies. The method of Good *et al.* (17) was used for extraction and hydrolysis of the glycogen. Glucose de-

terminations on the glycogen hydrolysates were carried out by the method of Somogyi (18) according to the colorimetric procedure of Nelson (19). Blood sugar determinations were performed by the same method. Values for six gold thioglucose-obese male Swiss mice are also included for comparative purposes. These mice were survivors from a group of Swiss mice which had been injected 6 months previously with 1 mg. of gold thioglucose per gm. of body weight (LD_{50}). The gold thioglucose was dissolved in water and injected intraperitoneally (20). At the time of injection the mice weighed between 20 and 25 gm. The average amounts of glycogen for the obese hyperglycemic and non-obese mice and for the gold thioglucose mice are given in Table I. They are expressed as mg. of glucose per 100 mg. of wet liver (per cent) and as mg. of glucose per total wet liver weight. The turnover of liver glycogen was measured by the amount

TABLE I

Levels of Liver Glycogen in Fed Obese Hyperglycemic Mice and Their Siblings and Gold Thioglucose-Obese Swiss Mice

Type of mouse	No. of mice	Body weight*	Liver glycogen*	Total liver glycogen*
		gm.	*per cent*	*mg*
Obese hyperglycemic	25	54 ± 8	3.72 ± 1.49	91 ± 49
Non-obese siblings	26	28 ± 5	3.20 ± 1.37	43 ± 22
Gold thioglucose-obese	6	60 ± 8	4.08 ± 1.13	89 ± 24

* Mean ± standard deviation.

of glucose-C^{14} incorporated into the liver glycogen. Each mouse received from a syringe pipette 0.25 cc. of an aqueous solution containing approximately 2×10^5 c.p.m. of uniformly labeled glucose-C^{14} by intraperitoneal injection at time zero and was killed by a blow on the back of the head after an interval of from 0.5 to 4.0 hours. The activity of the glucose-C^{14} was approximately 0.5 μc. per mg. The quantity of glucose-C^{14} injected was less than 1 mg. and did not significantly affect the blood sugar levels. The livers of the mice were quickly excised and weighed in tared Erlenmeyer flasks containing 5 cc. of 35 per cent KOH. After complete digestion the contents of the flasks were transferred quantitatively to 10 cc. volumetric flasks and made up to volume with 35 per cent KOH. 2 cc. aliquots of these digests were used for chemical determination of the amounts of glycogen. For the determinations of radioactivity 4 cc. aliquots of the KOH digest were used and the glycogen was purified by ethanol precipitation according to the method of Good *et al.* After the final precipitation the glycogen was dissolved in water and transferred quantitatively to 10 cc. volumetric flasks which were adjusted to volume with

water.   Duplicate 1 cc. aliquots of the glycogen solutions were pipetted into planchets and the glycogen was plated by evaporation of the water with an infra-red lamp.   The C^{14} radiation was analyzed with a thin end window Geiger counter.   All glycogen samples were counted for sufficient time to reduce the probable error to less than 5 per cent.   The activities per mg. of glycogen (specific activities) at assay periods of from 0.5 to 4.0 hours for the obese and non-obese mice are listed in Table II.   These values were corrected for the number of counts per minute of glucose-C^{14} injected,

TABLE II

Turnover of Liver Glycogen in Fed Obese Hyperglycemic Mice and Their Siblings As Measured by Incorporation of Glucose-C^{14}

Assay period	No. of mice	Activity per mg. glycogen*	Activity per gm. liver*	Activity per total glycogen*
		Obese		
hrs.		*c.p.m.*	*c.p.m.*	*c.p.m.*
0.5	3	4.7 ± 2.3	102 ± 37	220 ± 101
1.0	4	13.6 ± 12.7	191 ± 165	1024 ± 1189
2.0	5	28.9 ± 24.7	1193 ± 982	2910 ± 2652
3.0	7	60.9 ± 43.3	2933 ± 2547	5962 ± 4007
4.0	6	7.7 ± 4.3	231 ± 164	767 ± 883
		Non-obese		
0.5	3	3.1 ± 0.9	130 ± 35	156 ± 25
1.0	5	6.6 ± 4.6	289 ± 235	463 ± 439
2.0	6	22.5 ± 26.6	752 ± 953	762 ± 664
3.0	7	54.6 ± 66.9	1096 ± 1161	1283 ± 1294
4.0	5	7.2 ± 6.4	118 ± 98	145 ± 94

* Mean ± standard deviation.

for glycogen self-absorption, and dilution of the glucose-C^{14} by the blood sugar.   Inasmuch as the experiment extended over a period of several weeks, a number of different solutions containing only approximately the same activity of glucose-C^{14} per cc. were prepared and used.   The specific activities of these different glucose-C^{14} solutions were determined on an aliquot equal to that injected into each animal and a correction to a standard value of 2×10^5 c.p.m. injected was made.   Specific activities were corrected for glycogen self-absorption to a standard weight of 1 mg.   Finally, since the blood volumes of the obese and non-obese mice have been found to be equal (15) while the blood sugar levels are unequal, a correction in the specific activities for the dilution of the glucose-C^{14} by the blood sugars of the two groups of mice was also made.   The mean blood sugar

of a group of 107 obese mice was 178 ± 47 mg. per cent and the mean blood sugar for a group of 73 non-obese mice was 110 ± 19 mg. per cent. In addition to specific activity the results of the incorporation of glucose-C^{14} into glycogen were expressed as activity per total glycogen and activity per gm. of liver.

The linear regression coefficients listed in Table III were calculated from the individual values for glucose-C^{14} incorporation by the "method of least squares" with the exception of those for specific activity. These were calculated from the regression coefficients for total glycogen activity. The data for specific activity were not used because of the great variability in these values and the association in some cases of high specific activities

TABLE III

Regression Coefficients for Glucose-C^{14} Incorporation into Liver Glycogen in Obese Hyperglycemic Mice and Their Non-Obese Siblings

Type of mouse	Assay period	Activity per mg. glycogen		Activity per gm. liver		Activity per total glycogen	
		Slope*	"y" intercept	Slope*	"y" intercept	Slope*	"y" intercept
	hrs.	c.p.m. per hr.		c.p.m. per hr.		c.p.m. per hr.	
Obese ..	0.5–3.0	25.9 ± 14.2	−14.4	1218 ± 384	−890	2352 ± 700	−1306
"	3.0–4.0	−53.3 ± 17.9	220.8	−2702 ± 1049	11039	−5195 ± 1808	21551
Non-obese .	0.5–3.0	10.0 ± 8.7	−0.6	397 ± 192	−78	429 ± 193	−28
"	3.0–4.0	−47.4 ± 30.4	196.8	−981 ± 527	3794	−1139 ± 588	9

* ± standard error.

with high levels of total glycogen. Since the calculation of the slopes of the most probable straight lines required full use of all the data, a better statistical evaluation could be obtained by comparing the differences between the slopes than by comparing the differences between means at individual time periods. The latter embodied all points at only one time interval, while the former embodied all points at all time intervals studied.

The blood sugar and liver glycogen levels listed in Table IV were obtained from obese hyperglycemic and non-obese control mice, which were non-fasted and subjected to various periods of fasting. In addition one group of obese hyperglycemic mice was maintained *ad libitum* for a period of 2 weeks on a high fat carbohydrate-free diet previously described (11).

The levels of muscle glycogen given in Table V were obtained from a group of twelve mice consisting of equal numbers of male and female obese and non-obese mice. The animals had been maintained on laboratory

chow prior to the experiment. Glycogen determinations were performed on the hind leg muscles in each case.

Results

The levels of liver glycogen in the obese hyperglycemic and non-obese mice are listed in Table I. Comparative data for levels of liver glycogen are also listed for gold thioglucose-obese Swiss mice. These data revealed

TABLE IV

Effects of Carbohydrate-Free Diet and Various Periods of Fasting on Blood Sugar and Liver Glycogen Levels of Obese Hyperglycemic Mice and Their Non-Obese Siblings

Type of mouse	No. of mice	Treatment, hrs. fasted	Pretreatment blood sugar*	Posttreatment blood sugar*	Posttreatment liver glycogen*	Total posttreatment liver glycogen*
			mg. per cent	mg. per cent	per cent	mg.
Obese........	8	12	184 ± 51	134 ± 65	2.24 ± 1.12	36.2 ± 20.2
"	8	24	361 ± 94	184 ± 101	0.78 ± 0.42	14.6 ± 10.0
"	7	48	356 ± 88	151 ± 52	0.67 ± 0.52	11.2 ± 8.7
Non-obese...	8	24	152 ± 32	94 ± 34	0.20 ± 0.19	2.2 ± 2.3
Obese.....	6	CHO-free diet (11)†	236 ± 101	261 ± 115	4.37 ± 0.80	98.9 ± 23.9

* Mean ± standard deviation.
† Bibliographic reference No.

TABLE V

Level of Glycogen of Hind Leg Muscle of Fed Obese Hyperglycemic Mice and Their Siblings

Type of mouse (6 each)	Body weight*	Muscle glycogen*
	gm.	per cent
Obese....	58 ± 9	0.203 ± 0.041
Non-obese . .	32 ± 2	0.126 ± 0.044

* Mean ± standard deviation.

that, while the percentage of liver glycogen was similar for the obese hyperglycemic mice and their controls, the total quantities of glycogen were very dissimilar. The average liver weight for the twenty-five obese hyperglycemic mice was 2.45 gm. compared to 1.34 gm. for the non-obese mice. The mean liver weight of the six gold thioglucose-obese mice was 2.18 gm., a value similar to that found for the obese hyperglycemic mice. The average value of 89 mg. of total glycogen also compared closely with the average value of 91 mg. of total glycogen for the obese hyperglycemic mice. These results indicated that a greater functional liver mass and total glycogen were probably characteristic of obesity in general in mice and not a unique characteristic of the obese hyperglycemic mice. This is further substan-

tiated by the finding that a group of eight mice made obese by hypothalamic lesion with a mean body weight of 55 gm. had a mean liver weight of 2.23 gm. (21).

The results of the glycogen turnover study in the twenty-five obese hyperglycemic mice and the twenty-six non-obese siblings are given in Tables II and III. Inspection of the specific activity data in Table II indicated that both obese and non-obese mice reached a peak incorporation of glucose-C^{14} into liver glycogen at the 3 hour test period. The rate of incorporation decreased sharply after 3 hours and at the 4 hour period was comparable to the incorporation at 0.5 hour in the obese and at 1.0 hour in the non-obese. Comparison of the slopes of the specific activities for the obese and non-obese mice listed in Table III indicated that the rate of turnover of glycogen per molecule was 2.5 times as great in the obese as in the non-obese group.

Comparison of the activities in the total glycogen in the obese and non-obese mice listed in Table II showed that the obese animals incorporated considerably more glucose-C^{14} into liver glycogen per animal in all assay periods. Table III lists the slopes of the most probable linear values for these incorporations. From 0.5 to 3.0 hours the obese mice incorporated 2352 c.p.m. per hour compared to 429 c.p.m. per hour during the same time interval for the non-obese mice. The difference between these slopes was found to be highly significant ($p < 0.01$).

In view of the fact that the obese mice had a greater amount of total glycogen than the non-obese mice, it might be argued that they would be expected to show a greater incorporation of glucose-C^{14} per animal than the non-obese. To determine to what extent this increase in incorporation was dependent on liver size, the results were also expressed as activity per gm. of liver. The activities per gm. of liver at the various assay periods for the obese and non-obese mice are given in Table II. These results showed that, although the differences between activities per gm. of liver were not as great as those between activities per total glycogen, the obese mice still incorporated more glucose-C^{14} per gm. of liver than the non-obese mice at most assay periods studied. Comparisons of the slopes calculated for the most probable straight line for these incorporations per gm. of liver from 0.5 to 3.0 hours listed in Table III indicated that these slopes were significantly different ($p < 0.05$). It was inferred from these values of 1218 and 397 c.p.m. per hour per gm. of liver for the obese and non-obese mice, respectively, that the glycogen turnover per unit of liver weight, or alternatively per liver cell,[1] was 3 times as great in the obese as in the non-obese mice.

[1] Unpublished data of S. B. Andrus indicate that no significant difference in the size of parenchymal liver cells can be seen in histological comparisons of obese and non-obese liver cells.

The liver glycogen and blood sugar levels of obese mice maintained on carbohydrate-free regimens and various periods of fast are shown in Table IV. These data indicated that although carbohydrate alimentation was eliminated for a period of 2 weeks the obese mice were still able to maintain liver glycogen and blood sugar levels comparable to those seen in animals maintained on carbohydrate-containing chow diets. Inspection of the responses of the obese mice to various periods of fasting showed a progressive decrease in blood sugar and liver glycogen levels with increased duration of the fast. At the end of 48 hours of fasting the mean blood sugar level of the obese mice was in the range of the blood sugar level of fed non-obese mice. When comparisons were made between the obese and non-obese mice fasted for 24 hours, it was found that the blood sugar of the former group had decreased by 49 per cent and the latter group by 38 per cent.

The levels of glycogen in a group of representative skeletal muscles of the Bar Harbor mice are presented in Table V. Comparisons of the levels of muscle glycogen in the two groups showed that the obese mice had significantly higher ($p < 0.05$) levels than the non-obese.

DISCUSSION

The similar levels of blood sugar and liver glycogen maintained by the obese hyperglycemic mice when subsisting on either carbohydrate-containing or carbohydrate-free diets indicated that a dietary source of carbohydrate was not necessary for maintenance of blood sugar and liver glycogen in these animals. A consideration of these facts argued against the hyperglycemia of these obese mice being caused by their increased carbohydrate intake. This is further substantiated by the observation that hypothalamic obese mice (22) and gold thioglucose-obese mice (13), which are more hyperphagic than the genetically obese mice, are not hyperglycemic (22, 14).

The utilization of endogenous carbohydrate, especially the blood sugar, by the obese hyperglycemic mice during periods of fast was of interest. It indicated that, although the blood sugars normally exhibited by the obese mice were elevated when compared to their non-obese litter mates, homeostasis probably obtained at these hyperglycemic levels just as it did at the lower levels in the non-obese mice.

The significantly higher levels of muscle glycogen in the obese mice compared to their controls indicated certainly no impairment in their ability to store glycogen in the skeletal muscle mass. The greater amounts of glycogen exhibited by the obese mice may have been due to their greatly decreased spontaneous exercise (23). These results decreased the likelihood that epinephrine was involved in the diabetes of the obese mice. In hypersecretion of epinephrine one would expect lower than normal levels of

muscle glycogen. The effects of epinephrine on the obese mice will be discussed in more detail in a subsequent publication.

Since the primary endogenous source of the blood glucose is the liver glycogen, the turnover rates of glycogen would be expected to be intimately connected with the maintenance and turnover of these blood glucose levels. The greatly accelerated turnover of the total glycogen and glycogen per gm. of liver in the obese mice compared to that in the non-obese controls was, therefore, of particular importance. It has been shown above that about 6 times as many counts were incorporated in 2.5 hours in the obese as in the non-obese mice. The livers of the obese animals were about twice as large as those of the non-obese animals and the turnover of glycogen per gm. of liver was 3 times as great in the former group. Since the quantities of glycogen per gm. of tissue were similar in both groups, the amounts of glycogen at a cellular level were also similar. The synthesis and degradation of this amount of glycogen were therefore taking place 3 times as fast in the liver cells of the obese as in the liver cells of the non-obese mice.

The usual methods of calculation of rate of turnover and half life of metabolites cannot be applied here, as both incorporation and decay take place not against a constant level of labeled glucose, but against a decreasing gradient. Under the usual conditions with a constant concentration of label, incorporation and decay should take place at approximately the same rate. Under the conditions encountered here of a constantly decreasing concentration of label, it would be expected that incorporation which takes place while the amount of circulating glucose-C^{14} is high would cover a longer period than decay which takes place when the amount of circulating glucose-C^{14} is much smaller. This is in fact what was observed. In both obese and non-obese animals the rate of decay was approximately twice as great as the rate of incorporation.

While the classic equations do not apply in this case, it is still possible to calculate at least an order of magnitude of the rate of turnover. It has been observed that when 2×10^5 c.p.m. are injected, the obese animals incorporate a maximum of about 6×10^3 c.p.m. or 3 per cent of the total counts injected. The non-obese mice incorporate approximately 0.6 per cent of the total counts administered. As the weight of glucose injected was small enough to leave the blood sugars of fed animals unchanged, it may be legitimate to assume that the fate of these labeled molecules was representative of that of ingested glucose molecules. The obese mice consumed 6 gm. per day of a diet containing two-thirds of its calories as carbohydrate. If 3 per cent of the ingested carbohydrate was incorporated into liver glycogen, this would correspond to 120 mg. per day. Similarly, the non-obese mice who ate 5 gm. of this diet daily would have incorporated

20 mg. per day into their liver glycogen. On a per cent basis, the obese animals were turning over approximately 140 per cent of their liver glycogen per day, while the non-obese mice were turning over about 50 per cent. These figures correspond to 5 mg. and 0.83 mg. per hour being turned over by the livers of the obese and non-obese mice, respectively. This would correspond to 6 and 2 per cent, respectively, of the liver glycogen being turned over per hour and half lives of approximately 12 and 36 hours, respectively.

The values calculated for the half lives of liver glycogen are only rough approximations, since the assumption that the fate was the same for both intraperitoneally administered and orally ingested glucose was probably not strictly true. Further, handling and injecting the mice may have caused some epinephrine release which, due to its glycogenolytic effect, would decrease the incorporation of labeled glucose into liver glycogen. However, these limitations do not affect the validity of using the half lives for comparison purposes between the two groups. The value for the half life of liver glycogen in the non-obese mice was of the same order of magnitude as the half life of liver glycogen in rats reported by Stetten and Boxer. It appears from the findings on mice presented here and the results reported by Stetten and Boxer for rats that in fed animals the liver glycogen is relatively inert. During fasting, the liver glycogen is rapidly depleted to low levels, but as the fasting progresses the levels may be partially restored toward fed levels due to gluconeogenesis. If such conditions obtain, it might be predicted that fasting would cause an accelerated turnover of liver glycogen.

The marked differences between the diabetes of the obese mice and the diabetes due primarily to a lack of insulin are especially worthy of comment. In diabetes due primarily to a lack of insulin, such as that produced by destruction of the pancreatic β-cells with alloxan in experimental animals, there is both underutilization and overproduction of glucose (24–27). The results obtained on the obese mice presented a very different picture. The rapid decrease in blood sugar in response to fasting and the accelerated glycogen turnover indicated a faster than normal uptake of blood glucose. These factors, combined with the accelerated lipogenesis in these animals, indicated that there was actually overutilization of glucose in these animals in contrast to the underutilization observed in alloxan-diabetic animals. The maintenance of blood sugars of the same magnitude on carbohydrate-free diets as on diets containing carbohydrate indicated that there was also some overproduction of glucose. This overproduction probably was not too large, since it failed to prevent the blood sugar levels from decreasing during fasting. It is thus apparent that in these two types of diabetes, both characterized by hyperglycemia

and glycosuria, the metabolic characteristics are very dissimilar. Homeostasis in the metabolic processes regulating the blood sugar obtains in the obese hyperglycemic mice, while it does not obtain in the alloxan-diabetic animal. It is further evident that the metabolic picture in diabetes cannot be inferred from the blood sugar levels.

SUMMARY

1. The levels of liver glycogen per gm. of liver were similar in the obese hyperglycemic mice and their non-obese controls, but the former group had more than twice as much total glycogen. The greater total glycogen was due to the greater liver mass of the obese hyperglycemic mice. Mice, made obese by gold thioglucose, with livers of comparable size to those of the obese hyperglycemic mice also showed greater total glycogen levels.

2. The levels of glycogen in representative skeletal muscles were significantly higher in the obese hyperglycemic mice than in their non-obese controls.

3. The turnover of liver glycogen as measured by the incorporation of uniformly labeled glucose-C^{14} was approximately 6 times as great per total glycogen and 3 times as great per gm. of liver and per mg. of glycogen in the obese hyperglycemic mice as in their controls.

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